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Award Number: DAMD17-01-1-0371

TITLE: Breast Tumor/Stromal Cell Interaction in Bone

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REPORT DATE: July 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2004	Annual Summary (1 Jul 2001 - 30 Jun 2004)

4. TITLE AND SUBTITLE
Breast Tumor/Stromal Cell Interaction in Bone

5. FUNDING NUMBERS
DAMD17-01-1-0371

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract (Maximum 200 Words): We have investigated interactions between breast cancer and bone stromal cells and their role in regulating metastasis of breast tumor cells to bone and osteoclast development using an orthotopic mouse model. We found that PTHrP regulates tumor growth *in vivo* but is not required for specific metastasis of breast tumors to bone. Co-culture of bone stromal and tumor cells leads to up-regulation of RANKL and down-regulation of OPG, a response likely to contribute to breast metastasis to bone and osteolysis. The expression of RANKL and OPG *in vivo* is being investigated by immunohistochemistry using our orthotopic model. Experiments using an OPG-Fc fusion protein are ongoing but preliminary results suggest that adverse effects of this drug on lung metastasis may outweigh potential benefits for the treatment of bone lesions. Using *in vitro* and *in vivo* assays, we have demonstrated that stroma-derived MMP-9 plays a critical role in the development of lung and bone metastases. Results from co-culture experiments and immunohistochemistry suggest that tumor-derived MMP-9 may also contribute specifically to bone metastasis and osteolysis. This is being confirmed *in vivo* using MMP-9 null mice and RNAi technology to block tumor expression of MMP-9. Work investigating osteoclast requirement in tumor-mediated osteolysis using *in vivo* administration of OPG, β3 integrin peptidomimetics, and/or β3 integrin and M-CSF null mice is ongoing.

14. SUBJECT TERMS
Metastasis, breast cancer, mouse model, bone resorption, cell-cell interactions, cytokines, osteoclasts

15. NUMBER OF PAGES
41

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

Breast cancer is a leading cause of cancer mortality among western women. The high incidence of metastatic spread of breast tumors to bone in these women is, for the vast majority, associated with osteolytic lesions leading to significant clinical complications including osteoporosis, hypercalcemia, intractable pain, spinal cord compression and fracture of the long bones that invariably impair the quality of life of those affected (reviewed in (1)). Whilst the mechanisms leading to the preferential metastasis of breast cancer cells to bone remain poorly understood, recent findings from *in vitro* experiments using normal breast epithelial cells or tumor cells lines (2, 3) and the development of transgenic or xenograft animal models of breast cancer metastasis (reviewed in (4)) strongly support the critical role of the bone stromal microenvironment in the metastatic process and point to considerable cross-talk between bone cells and tumor cells leading to the establishment of secondary tumors in this organ. In particular, the use of an intracardiac injection model (5, 6) has been useful in unravelling the mechanisms and factors affecting bone remodelling and late stage progression of breast tumor metastasis to bone. However, whilst informative, current models are limited by the low incidence of spontaneous bone metastases and/or only allow the study of specific stages of the metastatic cascade.

We have developed an orthotopic mouse model of breast cancer metastasis to bone (7). In this model, tumor sublines of varying metastatic potential derived from a spontaneous mammary gland carcinoma from a Balb/cfC3H mouse are injected into an orthotopic site (mammary fat pad) of syngeneic animals. These animals consistently develop primary breast tumors that spontaneously progress to form metastases in bone and/or soft tissues (Figure 1). To improve our ability to measure quantitatively the extent of metastasis in defined organs, we have developed an assay based on real-time quantitative PCR (RTQ-PCR). By introduction of reporter genes into the tumor sublines, we have a marker to accurately measure metastatic tumor burden in any organ. This model has the advantage over existing models in that it mimics the entire metastatic cascade of the human disease, including the ability to evade immune surveillance. Importantly, the model enables us to study the interactions between stromal and tumor cells at both primary and metastatic sites in the same animal.

The overall objective of this project is to identify factors that regulate the establishment of breast cancer metastases in bone. Specifically, we aim to:

1- Determine the factors expressed by metastatic breast tumor cells and/or stromal cells in the bone microenvironment that regulate tumor cell growth and osteoclast activation.
2- Investigate the extent of osteoclast requirement in tumor-mediated osteolysis
3- Investigate the mechanisms of osteoclast activation by breast cancer cells

BODY

Task #1. To determine the expression of cytokines and osteoclast regulatory factors by breast tumor and bone stromal cells

The expression of osteoclast regulatory factors and cytokines by tumor cells has been implicated in the progression of bone metastases. Our orthotopic mouse model enables us to investigate in detail the expression of genes implicated in the literature to be important in the metastatic tumor environment, and to compare expression of these genes in the primary and secondary tumor sites. The expression patterns of selected molecules have been examined by reverse transcriptase real time quantitative PCR (RT-RTQ-PCR) immunohistochemistry (IHC) and immunofluorescence (IF).

PTHrP

The first gene taken for analysis this way was parathyroid hormone related protein (PTHrP). PTHrP has been implicated as a regulator of osteoclast activation, by upregulating expression of RANKL in osteoblasts (8). Over-expression of PTHrP in MDA-MB-231 cells enhances osteolytic activity in femurs after intracardiac injection of tumor cells into nude mice (9). However other data, both in animal models and clinical, cast doubt on the importance of PTHrP in regulating breast cancer metastasis to bone (10-12). Both primary tumors and metastatic tumor deposits of the bone metastasizing 4T1.2 tumor line immunostain strongly for PTHrP, as do some normal bone cells (Figure 2). Interestingly, the 66cl4 tumor line that does not metastasize to bone (see Figure 1) also stains strongly for PTHrP when grown as a primary tumor in the mammary gland. Further, when 66cl4 cells are injected directly into the tibia, they grow and express large amounts of PTHrP. These data suggest that PTHrP expression does not correlate with bone metastasis in our model. Further analysis of the role of PTHrP is described under Task 3.

Analysis of a panel of cytokines

We have also examined the expression of some other candidate genes, namely IL-1α, IL-1β, IL-6, IL-11, IL-17, IL-18, uPA, MMP2, MMP-9, RANKL, RANK and OPG by performing RT-RTQ PCR on RNA extracted from the primary tumors of the bone metastatic (4T1.2), the lung metastatic (66cl4) and the non-metastatic (67NR) tumors that comprise our model (Figure 3). This study provided an assay of differential gene expression between tumors of varying metastatic capacity and focussed our attention on IL-1α, IL-6, uPA, MMP2, MMP-9, RANKL and OPG for which the relative mRNA expression was highest in our tumor lines. Since the focus of this project is tumor/stromal cell interactions in bone, we have set up co-cultures of mouse osteoblasts and the tumor lines to gain further insight into the potential cross-talk between osteoblasts and metastatic tumor cells. Cultures of bone stromal cell populations enriched for osteoblasts were obtained from collaborative work with Dr Paul Simmons and Brenton Short from the Stem Cell Research Laboratory at Peter MacCallum Cancer Centre. The mRNA expression of RANKL, MMP-9, OPG and MT1-MMP as well as TGF-β was measured in these co-cultures by RT-RTQ-PCR and compared to the expression patterns in the lines grown separately. Whilst the relative expression of TGF-β was not altered by co-culture of tumor and bone stromal cells, we found a consistent up-regulation of RANKL mRNA expression in co-cultures of osteoblasts and the bone metastatic 4T1.2 cell line, in agreement with its role in osteoclast maturation and activation (Figure 4). Conversely, levels of the RANKL decoy receptor, OPG, were down-regulated in these cultures. These results strongly suggest that interactions between tumor and stromal cells may accelerate bone

resorption in the proximity of tumor deposits by shifting the balance towards pro-osteolytic factors.

MMP-9

In addition, MMP-9 mRNA expression was up-regulated and MT1-MMP was slightly down-regulated in co-cultures compared to mono-culture controls (Figure 4). The nature of these assays does not allow us to determine whether increased MMP-9 expression occurred in tumor cells, stromal cells or both. Current views on the role of MMP-9 in tumor invasion and metastasis suggest that MMP-9 and other metalloproteinases are secreted predominantly (but not exclusively) by stromal cells although the relative contribution of tumor- and stroma-derived MMP-9 in the metastatic process remains unclear. RT-RTQ-PCR analysis of MMP-9 expression in primary tumors revealed a significantly higher transcript level in bone metastatic cells (4T1.2) than in weakly (66cl4) and non-metastatic (67NR) lines (Figure 5A). This was confirmed at the protein level by immunoblot and gelatin zymography performed on *in vitro* cultures of the same lines (Figure 5B). None of the tumor lines expressed detectable levels of MMP-2 activity in the zymography assay. Thus, expression of MMP-9 (but not MMP-2) in our mammary carcinoma cell lines appears to correlate with bone metastatic capacity.

To address whether **tumor**-derived or **stromal cell**-derived MMP-9 protein expression was induced in co-cultures, we first generated subclones of the 4T1.2 cell line with reduced expression of MMP-9 through infection with a retroviral vector encoding a small inhibitory RNA (siRNA) sequence that specifically inhibits the expression of MMP-9 (pRs-MMP-9 siRNA). Control 4T1.2 cells were similarly generated using a vector encoding a siRNA directed against an irrelevant protein (Green Fluorescence Protein, pRs-GFP siRNA). Selection of infected 4T1.2 cells in the presence of puromycin gave rise to bulk cultures of 4T1.2-pRs-MMP-9siNNA and 4T1.2-pRs-GFPsiRNA that were then single cell cloned. Bulk cultures and clonal sublines of each 4T1.2 variant were tested for changes in MMP-9 expression by gelatin zymography (Figure 6). As expected, the parental 4T1.2 line expressed MMP-9 activity (lane 1) and expression level was not significantly affected in bulk cultures of 4T1.2-pRs-GFPsiRNA cells (lane 2). In contrast, MMP-9 activity was dramatically reduced in bulk cultures of 4T1.2-pRs-MMP-9siRNA cells (lane 3). Single cell clones isolated from these bulk cultures identified several clones with no detectable MMP-9 activity (lanes 7-9) whereas expression was maintained in clones of 4T1.2-pRs-GFP siRNA (lanes 4-6).

Selected clones were co-cultured with bone stromal cells derived from W/T and MMP-9 K/O animals. MMP-9 null C56BL/6J mice were acquired from Dr Vu, University of California and were backcrossed onto a Balb/c background in our laboratory to generate syngeneic W/T and K/O matched colonies. Following co-cultures experiments, supernatants analysed for gelatinolytic activity as described above (Figure 7). Consistent with the MMP-9 mRNA induction described above (see Figure 4), gelatinolytic activity detected in co-cultures of 4T1.2-pRs-GFP siRNA and W/T bone stromal cells confirmed the increased expression of MMP-9 activity (top left panel, lanes 5-6). The same experiment repeated using 4T1.2-pRs-MMP-9 siRNA cells failed to show any significant changes in MMP-9 activity (top right panel, lanes 5-6) suggesting that increased MMP-9 in tumor-bone stromal cell co-culture is derived from tumor cells. This was further demonstrated by the observation that increased MMP-9 activity occurred in co-culture of 4T1.2-pRs-GFP siRNA cells with MMP-9 null bone stromal cells (bottom left panel, lane 5-6). No MMP-9 activity was detected in mono- or co-cultures of 4T1.2-pRs-MMP-9 siRNA and MMP-9 null bone stromal cells (bottom right panel). Note also that MMP-2 was expressed by bone stromal cells irrespective of their MMP-9 status but its activity was not altered by co-culture with tumor cells. Taken together, these results clearly demonstrate that bone stromal cells up-regulate MMP-9

expression/activity in bone metastatic cells and suggest that this induction may contribute to the establishment and growth of breast tumor cells in bone.
These *in vitro* results have now been validated *in vivo* by IHC and RTQ-PCR performed on normal organs and spontaneous metastatic lesions derived from 4T1.2 mammary tumors in both W/T and MMP-9 K/O mice. These results and their significance with regards to the role of tumor vs stromal MMP-9 in breast cancer metastasis to bone are described under Task 3.

RANKL/OPG
We have devoted a considerable amount of time to analyzing the expression of RANKL protein *in vivo*. However, our attempts to demonstrate changes in RANKL expression in bone metastases by IHC using the only commercially available antibody reported to react with murine RANKL (R&D Systems cat#AF462) have been unsuccessful. After multiple interactions with Company representatives, we were recently informed that the antibody lot sent to us on two occasions was inactive (see attached appendix). These setbacks have to some extent delayed this aspect of the project but given the importance of these data for intended publications, the experiments will resume as soon as we receive a new antibody from R&D Systems and will be supported by internal funding. Further *in vivo* work addressing the role of OPG in bone metastasis is described in Task #2.

Chemokines and their receptors
In addition to the molecules originally proposed, we have measured the expression of a panel of chemokine receptors in the tumors. Chemokines have long been known to be important in the mobilization of immunological cells from thymus, spleen and bone marrow and their ability to home to sites of infection or damage. A recent study (13) highlighted the importance of chemokine Sdf-1 in the homing of breast tumor cells to specific sites, providing a possible mechanism whereby metastatic cells selectively colonize particular secondary organs depending on which chemokine receptor(s) they express. The receptor for Sdf-1 is CXCR4. It was therefore of great interest to determine whether chemokine driven migration may be responsible for the different patterns of metastasis in our model. The expression of a panel of chemokine receptors in the different tumor lines has been measured by RT-RTQ PCR (Figure 8). The chemokine receptors were found in varying levels in the tumors, with CCR1, CCR2, CCR5, CX3CR1 and CXCR4 showing the highest levels. However, expression of the ligands for these receptors is not confined to the sites to which the tumor cells metastasize (eg. bone and lung). To further this aspect of the work, we have set up a collaboration with Dr. Shaun McColl from Adelaide University, South Australia. Dr. McColl has a longstanding interest in chemokines and their function. Even though levels of expression of CXCR4 are low in cells of our metastasis model, bone is a major site of expression of its ligand Sdf-1 and thus could potentially contribute to homing of tumor cells to this organ (13). We have established that the 4T1.2 line has no endogenous expression of Sdf-1. These cells have been transfected with constructs encoding wild type Sdf-1 and a mutant form of Sdf-1 that acts as a CXCR4 antagonist. The aim is to determine whether the CXCR4/Sdf-1 axis is required for 4T1.2 metastasis to bone by disrupting the interaction with excess normal Sdf-1 or the receptor antagonist form. Preliminary results from these experiments indicate that exogenous expression of wild type sdf-1 but not mutant sdf-1 in the bone metastatic line 4T1.2 results in a significant reduction in primary tumor growth (Figure 9). We hypothesize that forced expression of w/t sdf-1 by tumor cells might induce infiltration of inflammatory cells causing an immune-mediated regression of the tumor. Organs are being processed for RTQ-PCR quantitative analysis of the effect of sdf-1 expression on metastatic tumor burden in bone and lung.

Task #2. To investigate the extent of osteoclast requirement in tumor-mediated osteolysis

Considerable evidence suggests that tumor cells cause bone destruction in part by recruiting osteoclasts (14). The role of osteoclasts in our mouse model of breast tumor metastasis to bone was examined using osteoclast inhibitors and mice lacking functional osteoclasts. We have used the osteoclast inhibitors, osteoprotegerin and bisphosphonates, the integrin β3 antagonist S247, a novel β3 inhibitor acquired from a pharmaceutical company and two strains of knockout mice with impaired osteoclast function – the m-CSF null mouse (op/op) and the integrin β3 null mouse.

1. Osteoprotegerin

Effect of enhanced tumor cell expression of OPG on metastasis to bone

Osteoprotegerin (OPG) is a decoy receptor for the osteoclast differentiation factor, RANKL and therefore competitively inhibits osteoclast activation by osteoblast and/or tumor cells (15). One of the advantages of our model is that we have developed an RTQ-PCR assay to measure metastatic tumor burden in bone and other organs. The 4T1.13 bone metastasizing clone was transfected with an expression construct for mouse OPG and single cell clones generated. The aim was to determine whether local expression of OPG in bone is effective in inhibiting osteolysis and therefore bone tumor growth. These clones were injected into mice either into the mammary gland or directly into bone. Following inoculation into the mammary gland, primary tumors generated from the control and OPG expressing lines developed at the same rate. However, tumor growth in the spine was reduced substantially (Figure 10). An *in vivo* assay of metastatic tumor growth, involving injection of tumor cells directly into the tibia, was used to determine the effect of tumor cell OPG expression on local growth in bone. Tumor cells overexpressing OPG showed reduced metastatic tumor burden compared to control (vector transfected) tumor cells 18 days after tumor inoculation (Figure 11). These data provide encouraging indications that osteolysis is an important factor in the regulation of tumor cell growth in bone, probably via the release of stimulatory cytokines.

Treatment of bone metastasizing tumors with recombinant OPG

Mice were injected into the mammary gland with the bone metastasizing clone, 4T1.13. Once the tumors were palpable, the mice were treated with daily injections of recombinant Fc-OPG (kindly supplied by Amgen) at 1 mg/Kg/day. (Figure 12). In two separate experiments, the spines of mice treated with Fc-OPG displayed reduced metastatic tumor burden compared to controls. These results border on statistical significance and further experiments are required to validate these results. Nevertheless, the data suggest that metastatic tumor growth in our model is at least partially dependent on osteolysis. Amgen have recently supplied us with a new version of OPG, (OPG-Fc) which is more potent and has a longer half-life in mice. We have undertaken experiments to test the efficacy of this new OPG-Fc fusion protein in our mouse model of spontaneous metastasis model.

For initial experiments, the drug was administered as recommended by Amgen and consisted of twice weekly injection of 5 mg/kg intra-peritoneal starting on day 7 post-inoculation of tumor cells into the mammary fat pad. However, on the 4th administration of the drug, all mice suffered anaphylactic shock within 10 min following injection and died of respiratory distress. Blood samples from these mice were collected and analysed by ELISA for the presence of antibodies directed against the fusion protein (Figure 13). As expected, drug-treated but not control mice had a high titer of anti-OPG-Fc antibodies confirming that the

mice developed an immune reaction against the drug. We believe that this is likely to be due to species specificity and reactivity against the human Fc portion of the fusion protein. To circumvent this problem, we devised a new protocol whereby the drug administration was limited to 3 IP injections starting 7 days post-tumor cell injection (early intervention) or starting 14 days post-tumor cell injection (late intervention). Under these conditions, all mice survived the treatment enabling measurement of primary tumor growth and RTQ-PCR analysis of metastatic burden in lung, spine and femurs harvested on day 25. As shown in Figure 14, OPG-Fc had no significant effect on primary tumor growth rate (panel A) or final tumor weight at completion of the experiment (panel B). Surprisingly, analysis of tumor burden in lung (Figure 15) revealed a dramatic increase in tumor burden in the drug-treated group and particularly evident in the early intervention mouse group. In contrast, tumor burden in femurs and spine, was low in control mice and not significantly affected by administration of OPG-Fc under either protocol. This is consistent with the absence of significant changes in plasma calcium levels, an indicator of bone osteolysis associated with metastatic growth in bone (Figure 16). It remains unclear whether the administration of OPG and/or an immune reaction to the Fc-fusion protein contributed directly to the increase tumor metastasis to lung. We hypothesize that the high tumor burden in this organ necessitated early termination of the experiment due to signs of distress noted in several animals (day 25 instead of day 28-30) and consequently resulted in low tumor burden in bone even in control animals. To address these issues, we intend to test the effect of OPG-Fc in immunocompromised mice. Also, we have initiated studies using mouse RANKL-Fc fusion protein (provided by Amgen) instead of OPG-Fc, where development of an immune response will not be an issue (mouse OPG-Fc is not available in sufficient quantities).

2. Bisphosphonates

Bisphosphonates are used clinically to alleviate bone pain caused by malignant disease. They are able to coat the bone surfaces with a layer resistant to attack by osteoclasts. They have also been shown to induce apoptosis in osteoclasts. We have tested Pamidronate and Zoledronate several times for their ability to protect mice from osteolytic damage caused by the bone metastasising lines 4T1.2 and 4T1.13. We have never seen a reduction in bone tumor burden using these agents and thus our investigation will not be pursued.

3. *S247*

S247 is a peptidomimetic antagonist for the integrin β3, being developed by Pharmacia. Experiments testing whether S247 can block metastasis to bone by disrupting the function of the integrin β3 have been performed but have necessitated early termination of treatments due to the development of fever (hot ears) in some mice. We have now initiated experiments with another novel β3 inhibitor obtained from another pharmaceutical company. Whilst we have not observed any adverse side effects in treated mice, the proprietary nature of this compound prevents us from commenting further on the results of these experiments.

4. *β3 integrin*

Mice lacking β3 integrin display an osteopetrotic phenotype due to the lack of functional osteoclasts (16). These mice are being backcrossed onto a Balb/c background. We have completed 10 backcrosses and have verified normal tumor growth in β3 integrin wild type offspring. Heterozygote mating has been initiated and is being expanded to obtain sufficient

K/O and W/T littermates for the proposed experiments. 4T1.2 cells will be injected into the mammary gland and growth of the primary tumor and metastasis to lymph nodes, lung and bone will be assessed. To complement this work we have also addressed the role of tumor β3 integrin in breast cancer metastasis by stable expression of a β3 construct in non-bone metastatic cells (66cl4). The results of our investigation have been submitted for publication and demonstrate that expression of β3 integrin in tumor cells enhances their metastasis to lung and promotes metastasis to bone by facilitating interaction with extracellular matrix proteins present in the bone stromal microenvironment.

5. *M-CSF*

M-CSF (op/op) mice also display an osteopetrotic phenotype due to the lack of m-CSF required for osteoclast activation. These mice have been backcrossed onto the Balb/c background as well. They have been backcrossed ten times and normal growth of the 4T1.2 tumor line has been demonstrated in the wild type offspring. However, generation of wild type and null mice for experiments has been slowed considerably due to low birth rate and frequent early death of the null offspring. New breeding pairs have been set up and approximately 10-12 births have been obtained recently which should able us to initiate these experiments.

Task #3. To examine mechanisms of osteoclast activation by tumor cells

The mechanism(s) by which tumor cells induce osteolysis are not well understood and the identification of factors important in this process is a major focus of this project. The role of OPG in osteolysis induced by metastatic tumor cells is being investigated as described in Specific Aim 2. In this section, we are investigating the role of factors identified above to promote or inhibit osteolysis. In addition, the role of matrix metalloproteinases in our model is being examined by backcrossing MMP12 and MMP-9 knockout mice onto the Balb/c background. The MMP12 knockout mice have been backcrossed ten times and normal tumor growth in β3 integrin wild type offspring has been confirmed. These mice are being expanded to generate sufficient wild type and null mice for experiments. This step has already been completed for the MMP-9 mice and considerable experimental data have been obtained. This is described below.

PTHrP

As mentioned above, there are considerable data suggesting that PTHrP is important in breast cancer metastasis to bone. We have investigated the role of PTHrP in our model of metastasis by genetically engineering altered PTHrP expression levels in tumor cell lines. Our data show that overexpression of PTHrP does not enhance the ability of tumor cells to metastasize to bone. However, inhibition of PTHrP expression using antisense cDNA constructs reduces the growth rate and therefore metastatic tumor burden of 4T1.2 tumor cells (Figure 17). However, when the tumors bearing the PTHrP antisense construct were grown for an extra week so that the primary tumors are the same size as those of the control group, there was no reduction in tumor burden in bone (Figure 18). These data demonstrate that PTHrP is required for tumor cell growth and that antisense PTHrP may block this intracellular function of PTHrP. However, it is not required for metastasis to bone.

MMP-9

Matrix metalloproteinases (MMPs) have been implicated in the metastatic progression of many cancers. However, whilst MMPs have been postulated to contribute to increased migration and invasion of breast tumor cells, their precise role in the metastasis of breast tumor cells to bone is incompletely understood (17). High serum expression of MMP-2 and MMP-9 has been reported in breast cancer patients and high MMP-9 activity levels in plasma of breast cancer patients correlates with a poorer overall survival rate (18, 19). Moreover, Ohshiba et al. (20) reported that co-culture of MDA-MB-231 breast cancer cells with bone calvaria increased bone resorption and this response was associated with increased expression of pro-MMP-2, active MMP-2, pro-MMP-9 and MMP-13. These results implicate multiple MMPs in the osteolytic response associated with breast cancer metastasis to bone. We have reported that the bone metastatic cell line 4T1.2 is more invasive in *in vitro* assays and secretes higher levels of MMP-9 than the weakly metastatic (66cl4) and non-metastatic (67NR) cell lines ((21) and see Figure 5). The increased expression of MMP-9 in co-cultures of 4T1.2 and bone stromal cells discussed above (see Task #1) indicates that expression of tumor-derived MMP-9 may be regulated by bone stromal cells. We have extended our *in vitro* findings by performing immunohistochemical (IHC) analysis of MMP-9 in our *in vivo* orthotopic model of breast cancer metastasis to bone.

We last reported that examination of normal femur sections from wild type mice by IHC revealed high levels of MMP-9 in the bone marrow and at sites of active remodeling (growth plates) (Figure 19). Further analysis of femoral metastases derived from mammary fat pad injection of 4T1.2 cells demonstrated scattered MMP-9 positive cells within the tumor mass and high MMP-9 particularly evident at the periphery of the tumor in close contact with the bone stroma and active sites of bone resorption by osteoclasts (Figure 20). A similar pattern of MMP-9 reactivity was detected following direct intratibial injection of 4T1.2 cells in wild type mice (Figure 21). Interestingly, when direct intratibial injections were performed in MMP-9 deficient animals, intense staining of the entire tumor area was detected whereas, as expected, the unaffected marrow showed no MMP-9 reactivity (Figure 22).

Taken together, these results illustrate the interplay between tumor and bone stroma and suggest that both stroma and tumor-derived MMP-9 may be critical for the establishment of breast cancer metastasis to bone. This is consistent with the results from co-culture experiments described above (see Figure 7). However, the possibility that trauma resulting from the intratibial injection procedure may in part contribute to the increased MMP-9 expression detected in the tumors of MMP-9 null mice could not be completely ruled out. To address this and to determine if the development of spontaneous metastasis in these animals is also associated with increased MMP-9 expression in bone metastases, we have now extended our IHC observations to spontaneous lung and bone metastases arising from fat pad injection of 4T1.2 cells in MMP-9 deficient and wild type mice.

Normal non-tumor bearing wild type Balb/c mammary glands express very low levels of MMP-9 in the surrounding stroma (Figure 23). A greater number of MMP-9 expressing cells were detected in normal lung and were found scattered throughout the lung and most likely representing alveolar macrophages. MMP-9 was also abundant in the marrow of femurs and spine. As expected, no MMP-9 reactivity was detected in any organs of MMP-9 null mice (data not shown). A similar analysis of tumor-bearing lung and spine of wild type and MMP-9 K/O mice revealed interesting findings. In the lung, MMP-9 was abundant and its expression particularly high in the stroma directly in contact with the metastatic lesions whereas scattered MMP-9 positive cells were found in the metastatic deposit. In contrast no MMP-9 was detected in the stroma or tumor foci in lung metastases of MMP-9 K/O animals. These results suggest that MMP-9 positive cells found within the lung metastatic deposit in

wild type mice are infiltrating stromal cells and that tumor cells in this organ do not express detectable MMP-9. A different conclusion emerged from our analysis of spine metastases. MMP-9 expressing cells were found scattered throughout the tumor lesion and abundant in the adjacent stroma in wild type animals. These observations were similar to that made in wild type lung. However, tumor cells in spine metastases of MMP-9 K/O mice expressed high levels of MMP-9 whereas the surrounding stromal cells were negative. These results are consistent with our *in vitro* findings from co-culture experiments and indicate that unlike lung metastases, expression of MMP-9 in bone metastatic cells is induced by surrounding stromal cells.

To address the significance of these observations, we have performed RTQ-PCR analysis of metastatic burden in lung, spine and femurs of W/T and MMP-9 K/O mice following injection of 4T1.2 cells in the mammary fat pad. As shown in Figure 24, primary tumor weight was not significantly different between W/T and K/O groups (Figure 24a). However, tumor burden in lung ($p< 0.01$) and femur ($p< 0.05$) was dramatically decreased in MMP-9 K/O mice (Figure 24b). A similar trend was observed in spine but did not reach significance. Organs from twenty additional mice (10 × K/O + 10 × W/T) have been harvested and are being processed for RTQ-PCR analysis. These data clearly demonstrate that stroma-derived MMP-9 plays a critical role in spontaneous metastasis of breast tumor cells to lung and bone.

As discussed above, we have generated stable 4T1.2 sublines that lack expression of MMP-9 through infection with a pRs-MMP-9 siRNA or control vector. At least 5 single cell clones of 4T1.2 pRs-MMP-9 siRNA with no detectable MMP-9 activity and 5 control clones of 4T1.2-pRs-GFP have been identified. These have been pooled and will be used for measurement of metastatic burden in W/T and MMP-9 K/O mice. The combination of RNAi methodology and MMP-9 null mice will clarify whether tumor-derived MMP-9, stroma-derived MMP-9 or both contribute to the establishment of breast cancer metastases in lung and bone and associated bone resorption. Given our IHC observations that 4T1.2 tumor cells express MMP-9 in bone but not lung metastases, we hypothesize that specific inhibition of MMP-9 in tumor cells by siRNA will further reduce tumor burden in bone but not in lung.

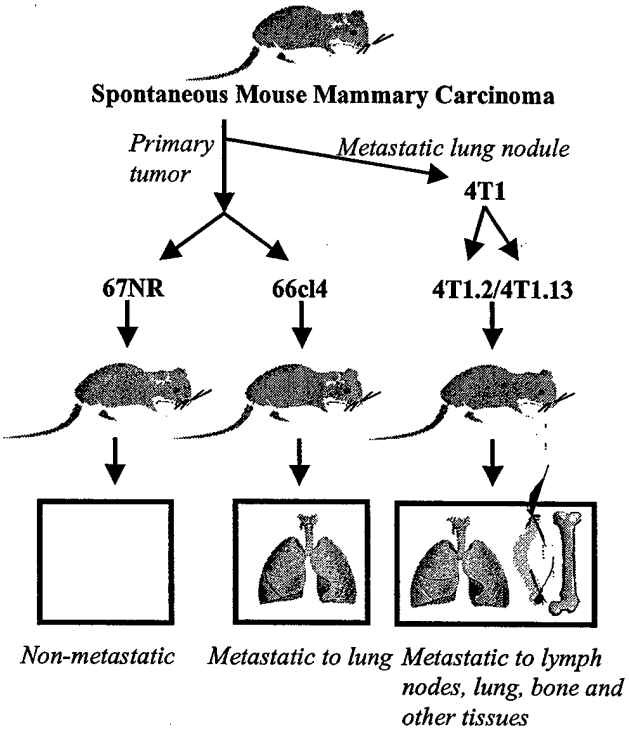


Figure 1. Orthotopic model of breast cancer metastasis. Several tumor sublines have been isolated from a spontaneously arising mammary gland carcinoma. Each subline has a distinct metastatic phenotype. 67NR is non-metastatic, whereas 66cl4 is weakly metastatic to lung. 4T1.2 and 4T1.13 are two bone metastazising tumor clones derived from the lung metastasizing 4T1 tumor subline isolated from a metastatic lung nodule.

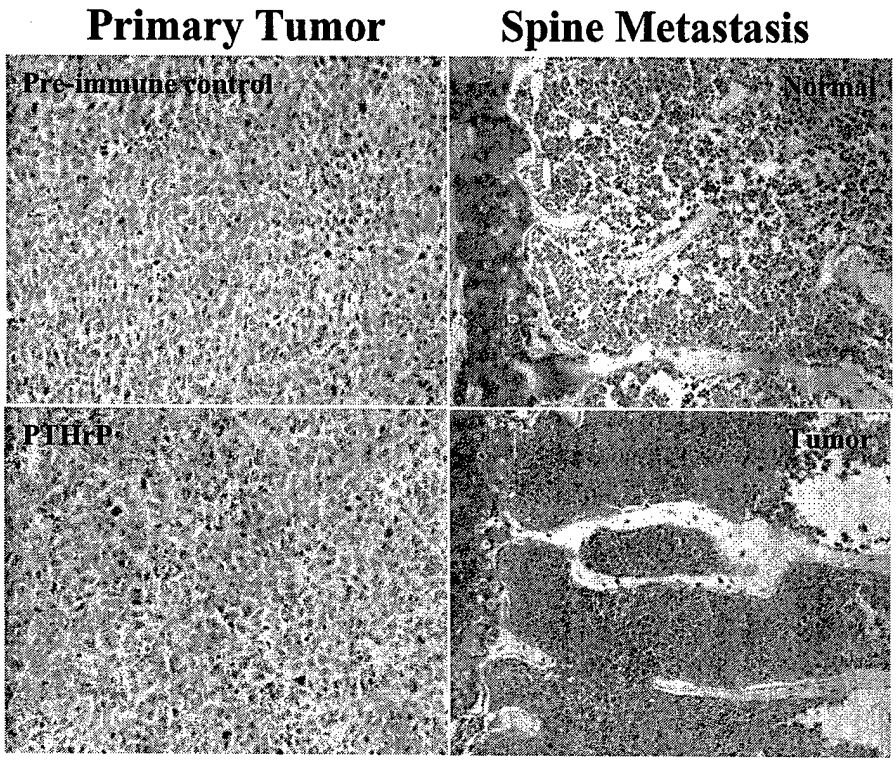


Figure 2. Immunohistochemical detection of PTHrP in primary mammary tumor and bone metastasis. Sections were prepared from primary tumor and spine of mice bearing 4T1.2 tumor, stained with hematoxylin and eosin and immuno-stained with a peroxidase-conjugated anti-human PTHrP antibody. Top left: section through primary tumor labelled with control pre-immune serum. Bottom left: section of primary tumor labeled with specific antibody directed against PTHrP. Top right: section of normal bone and bone marrow labelled with PTHrP antibody. Bottom right: adjacent section of spine showing large tumor deposit expressing PTHrP.

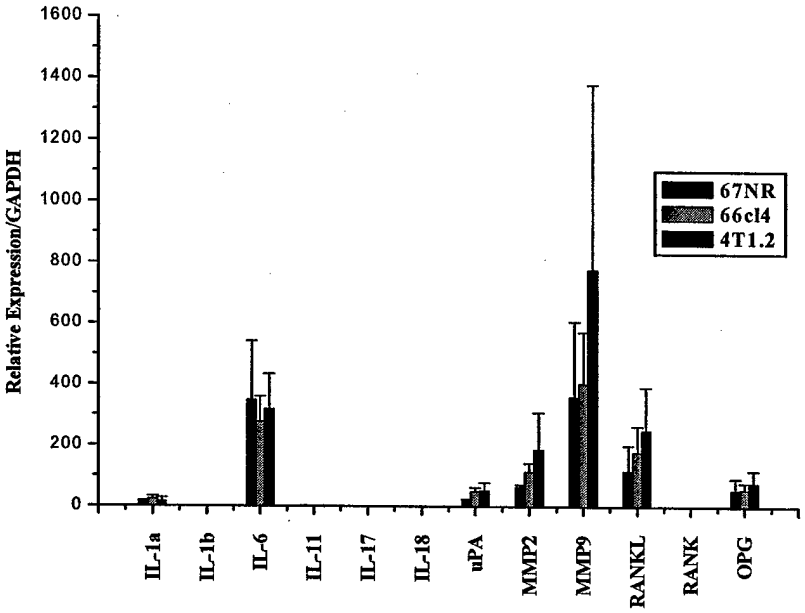


Figure 3. Expression of candidate genes in primary tumors. RNA was extracted from three primary tumors for each of the tumor lines indicated. cDNA was synthesized and SyBr Green RT-RTQ-PCR performed for each of the genes indicated. Expression relative to GAPDH was determined and error bars represent the standard error of the mean.

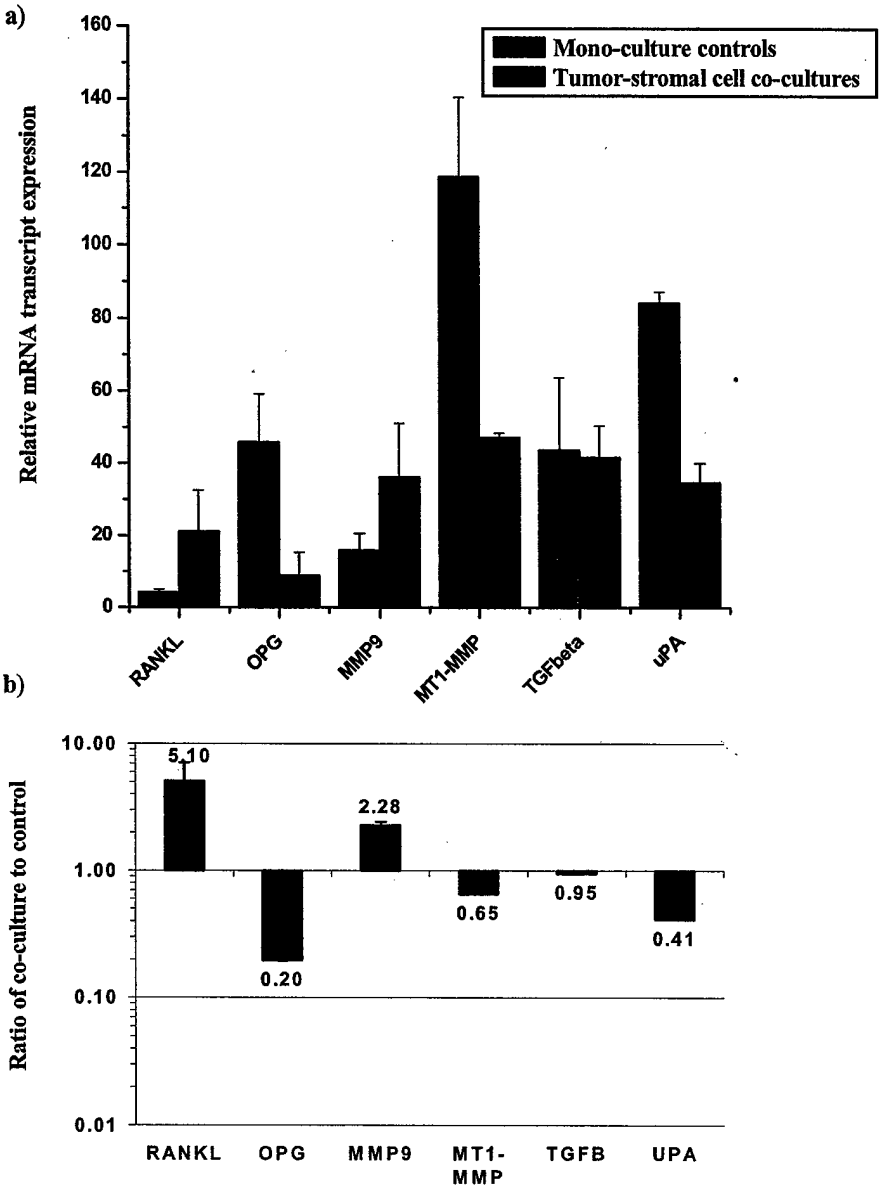


Figure 4. Regulation of gene expression by co-culture of tumor and stromal cells in vitro. RNA was extracted from control tumor and stromal mono-cultures or co-cultures of 4T1.2 tumor cells and bone stromal cell population enriched for osteoblasts. CDNAs were synthesized and SyBr Green RT-RTQ-PCR performed for each of the genes indicated. (a) mRNA transcript expression relative to GAPDH was determined and the results represent the means ± SD of triplicates. (b), the results from panel are expressed as a ratio of mRNA expression of co-cultures/control mono-cultures to reflect the changes in transcript abundance for co-cultures of tumor-stromal cells.

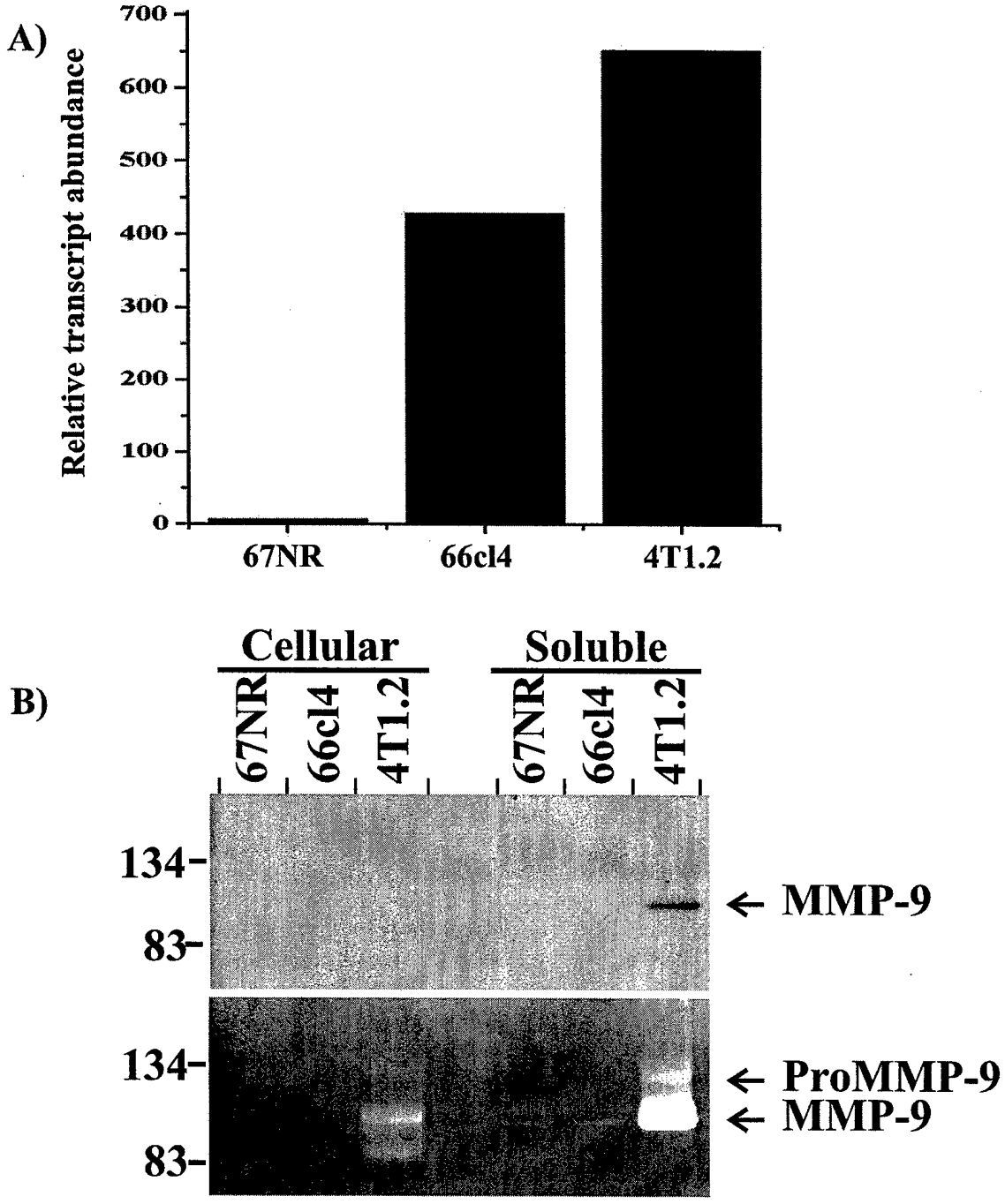


Figure 5. Expression of MMP-9 in primary breast tumors and in *in vitro* cultures of 67NR, 66cl4 and 4T1.2 cell lines. The expression of MMP-9 mRNA in primary tumours following injection into the mammary fat pad of syngeneic mice was analysed by RTQ-PCR (panel A). Secreted and cell-associated MMP-9 protein from tumor cell mono-cultures was detected by western blotting (panel B, top) and gelatin zymography (panel B, bottom). Note that the highly metastatic cell line 4T1.2 expressed higher level of MMP-9 than the weakly (66cl4) and non-metastatic (67NR) lines both *in vivo* and *in vitro* and that the majority of MMP-9 in vitro was detected in the culture medium in its active form.

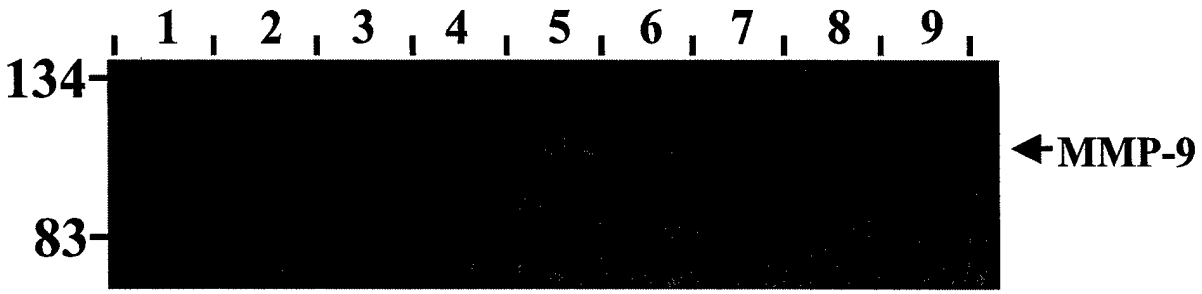


Figure 6. Detection of gelatinase activity in 4T1.2 pRs-GFP and 4T1.2 pRs-MMP-9 siRNA tumor lines. 4T1.2 cells were infected with a retroviral vector encoding siRNA directed against a control irrelevant protein (green fluorescent protein, GFP) or MMP-9 and infected cells selected in the presence of puromycin. Bulk cultures were single cell cloned and supernatants from bulk cultures and selected clones tested for gelatinolytic activity by gelatin zymography. Lane 1, uninfected 4T1.2 cells; lane 2, 4T1.2 pRs-GFP siRNA bulk cultures; lane 3, 4T1.2 pRs-MMP-9 bulk cultures; lanes 4-6, 4T1.2 pRs-GFP clones; lanes 7-9, 4T1.2 pRs-MMP-9 siRNA clones. Note the significant reduction in MMP-9 activty in the culture supernatant of 4T1.2 pRFs-MMP-9 bulk cultures (lane 3) and complete absence of detectable activity in selected MMP-9 siRNA clones (lanes 7-9).

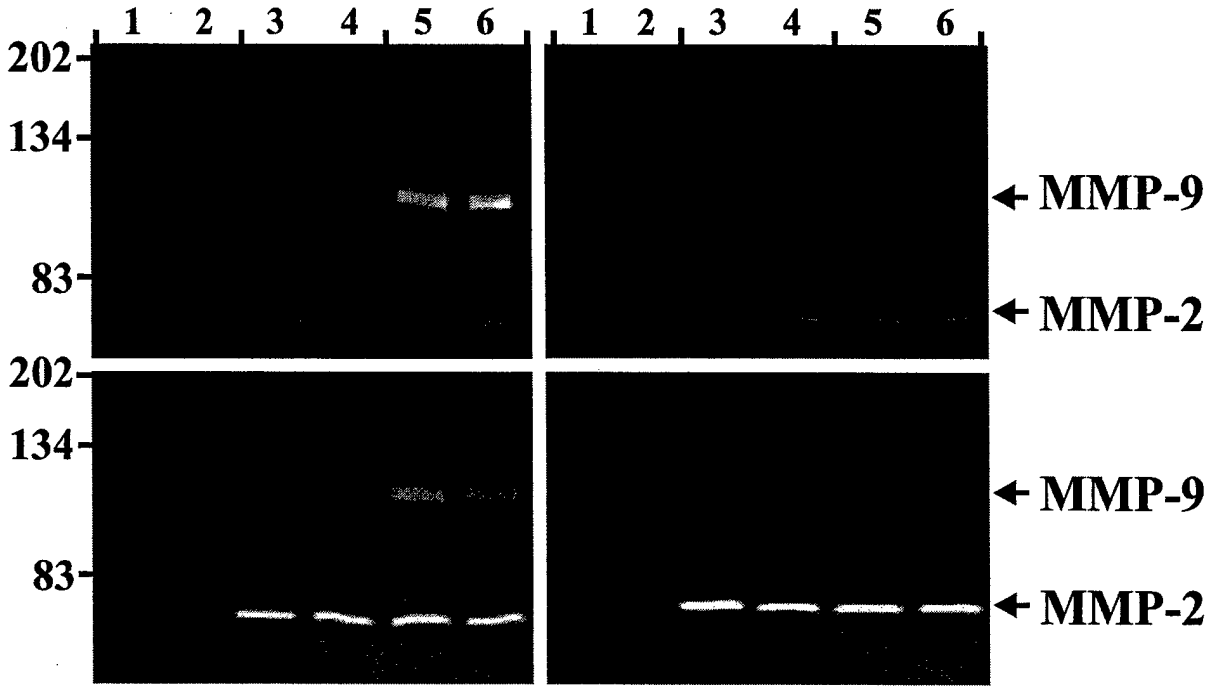


Figure 7. Induction of tumor-derived MMP-9 activity by bone stromal cells. Bone stromal cells were harvested from collagenase digests of tibias and femurs derived from wild type and MMP-9 null mice and expanded *in vitro*. Clones of 4T1.2 pRs-GFP siRNA (control) and 4T1.2 pRs-MMP-9 siRNA were seeded in serum-free medium and cultured as mono-cultures (1 x10^5/100ul/well) or co-cultures with wild type (top panels) or MMP-9 null (bottom panels) bone stromal cells (1x10^5/well) for 24h. Supernatants from duplicate mono and co-cultures were tested for the presence of gelatinolytic activity by standard gelatin zymography. Top left panel: 4T1.2 pRs-GFP alone (lanes 1-2); wild type bone stromal cells alone (lanes 3-4); 4T1.2 pRs-GFP + wild type bone stromal cells (lanes 5-6). Note the strong up-regulation of MMP-9 in co-culture supernatants. Top right panel: 4T1.2 pRs-MMP-9 siRNA cells alone (lanes 1-2); wild type bone stromal cells alone (lanes 3-4). ; 4T1.2 pRs-MMP-9 siRNA + wild type bone stromal cells. Note the absence of MMP-9 up-regulation when 4T1.2 pRs-MMP-9siRNA cells are used for co-culture. Bottom left panel: 4T1.2 pRs-GFP siRNA cells alone (lanes 1-2); MMP-9 null bone stromal cells alone (lanes 3-4); 4T1.2 pRs-GFP siRNA + MMP-9 null bone stromal cells (lanes 5-6). Bottom right panel: 4T1.2 pRs-MMP-9 siRNA cells alone (lanes 1-2); MMP-9 null bone stromal cells alone (lanes 3-4); 4T1.2 pRs-MMP-9 siRNA + MMP-9 null bone stromal cells (lanes 5-6). Note the up-regulation of MMP-9 actvity detected in co-cultures of MMP-9 null bone stromal cells with 4T1.2 pRs-GFP siRNA (left panel) but not with 4T1.2 pRs-MMP-9siRNA cells (right panel) confirming that increased MMP-9 activity is derived from tumor cells rather than bone stromal cells.

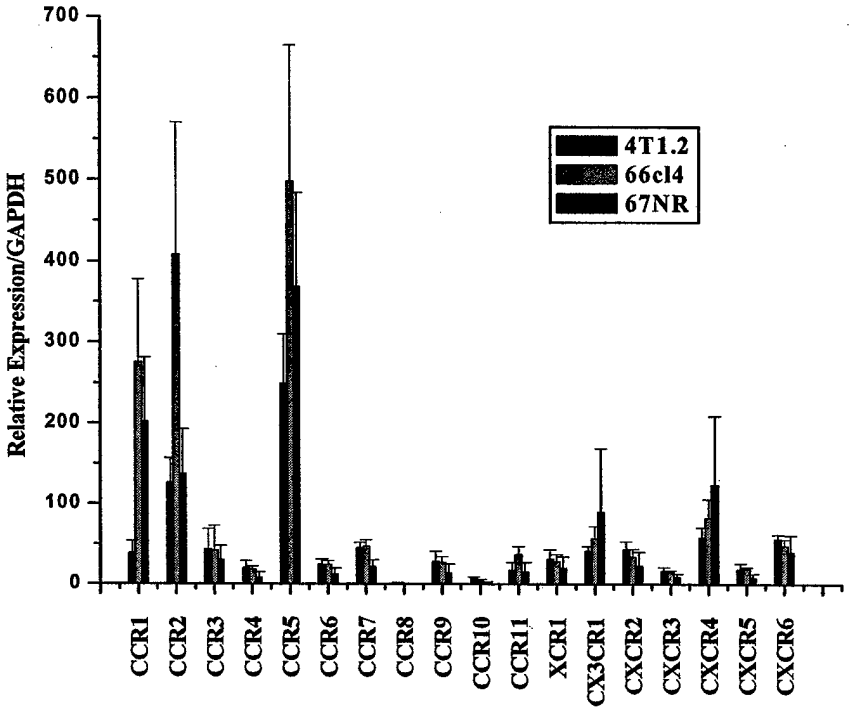


Figure 8. RT-RTQ-PCR Analysis of Chemokine Expression in Tumor Cells. RNA was extracted from three separate primary tumors for each of the tumor lines indicated. cDNA was synthesized and SyBr Green RT-RTQ-PCR performed for each of the chemokine receptors. Expression relative to GAPDH was determined and error bars represent the standard error of the mean.

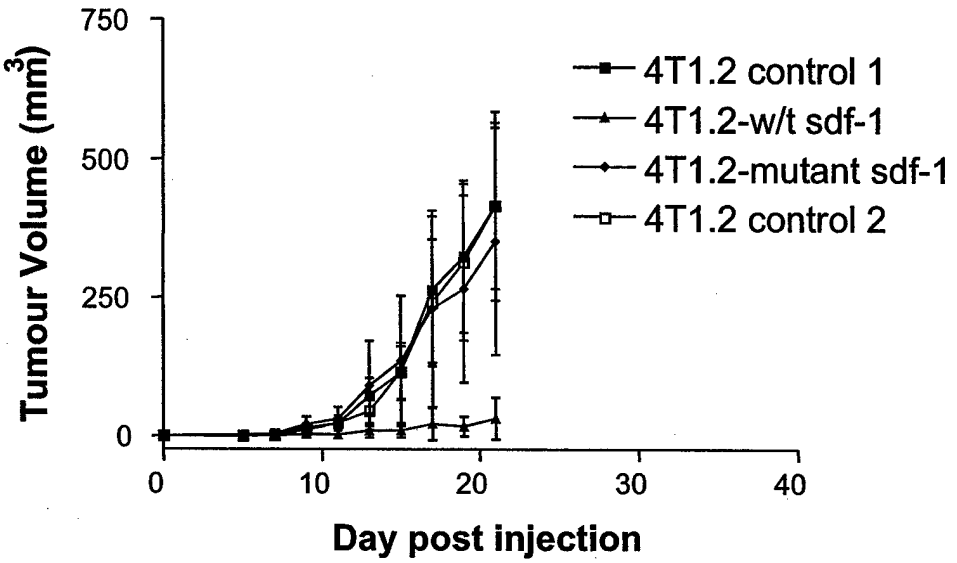


Figure 9. Inhibition of primary tumor growth by overexpresssion of wild type sdf-1 in 4T1.2 cells. 4T1.2 cells were transfected with a vector encoding wild type sdf-1or mutant form of sdf-1 and injected into the mammary fat pad of Balb/c mice. Tumor volume was monitored with electronic calipers every 2 days up from day 5 to day 21. Data represents means+/- SEM of 5 mice per group. Note the dramatic reduction of growth in 4T1.2 tumors overexpressing w/t sdf-1 but not the mutant form of sdf-1.

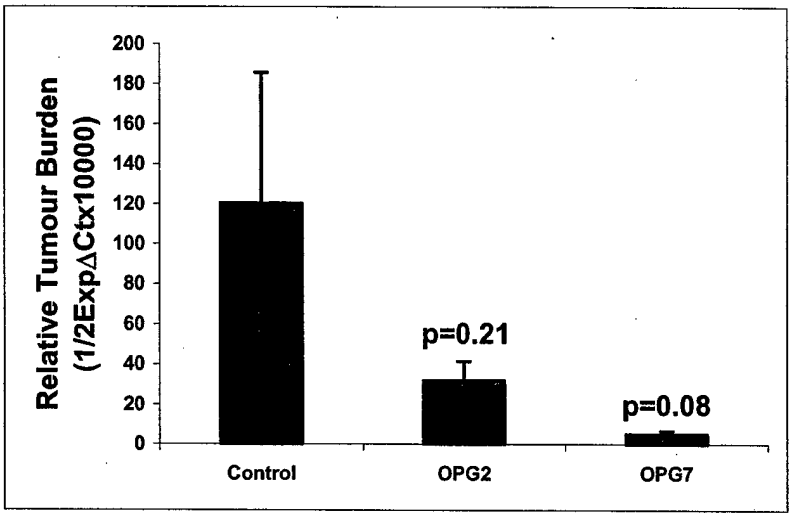


Figure 10. Overexpression of OPG in Breast Tumor Cell Lines Reduces their Bone-Metastatic Potential. Mice (n = 15) were inoculated into the mammary gland with 4T1.13 base vector (control) or with one of two OPG-expressiong clones (OPG2 and OPG7). Primary tumors were allowed to develop for 30 days. Genomic DNA was prepared from spine and RTQ-PCR used to determine metastatic tumor burden. Error bars represent standard errors of the mean and p values were determined using the student t test.

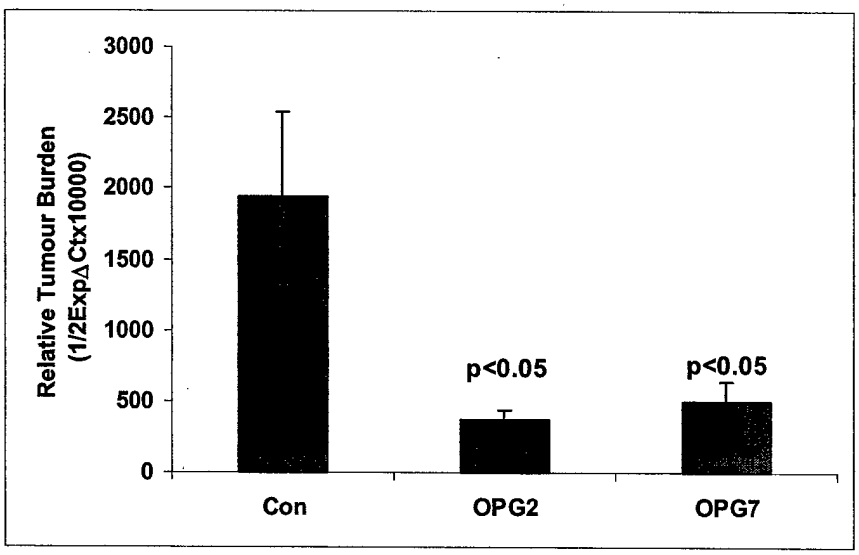


Figure 11. *In Vivo* Growth of Tumor Cells Overexpressing OPG. Two single cell clones overexpressing OPG and one base vector transfected clone of 4T1.2 were injected directly into the mouse tibia (n = 8) and tumor cells allowed to grow for 18 days. Tibia were removed and tumor growth measured using RTQ PCR. Error bars represent standard error of the mean and p values were determined using the student t test.

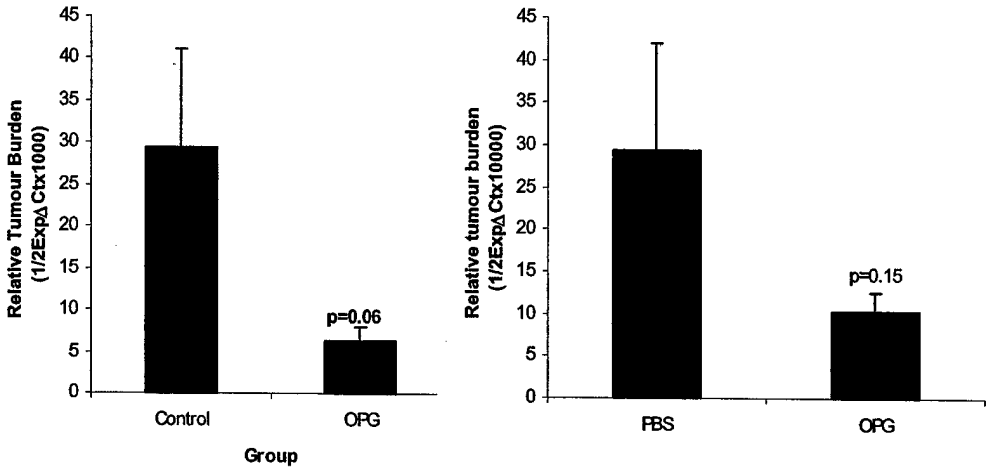


Figure 12. Effect of Fc-OPG on Metastatic Tumor Burden in Spine. Mice (n = 15) were injected with 4T1.13 and primary tumors allowed to develop for either 11 days (panel a) or 14 days (panel b). Fc-OPG (1mg/kg/day) was then administered daily by subcutaneous injection for the next 14 days. Genomic DNA was prepared from spine and RTQ PCR used to determine metastatic tumor burden. Error bars represent standard errors of the mean and p values were determined using the student t test.

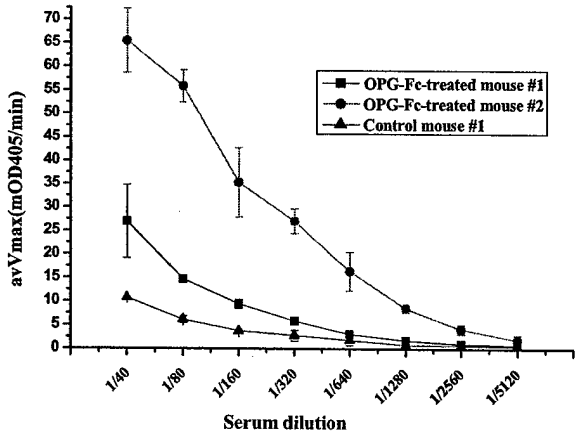
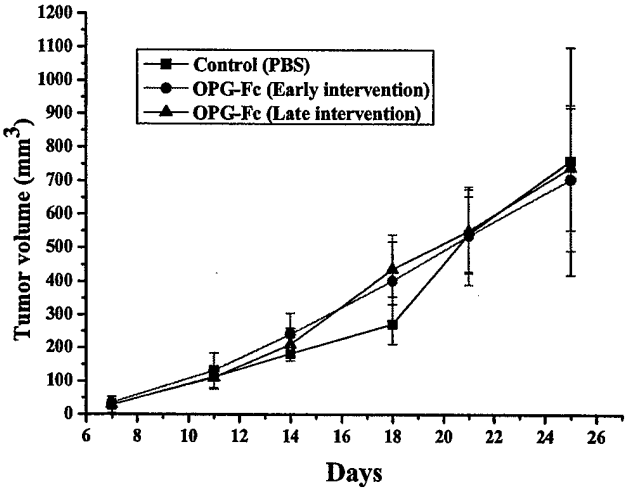


Figure 13. Detection of OPG-Fc reactive antibodies in the serum of OPG-Fc-treated mice. Balb/c mice were inoculated with 4T1.2 cells (1 x 10^5) into the mammary fat pad and treated twice weekly with intraperitoneal injection of OPG-Fc (5mg/Kg) or PBS (control) starting on day 7. Following the 4^{th} injection, all OPG-Fc-treated mice died of respiratory distress. Blood samples were collected from control (PBS) and OPG-Fc-treated mice and the serum analyzed for the presence of OPG-FC-reactive antibodies by ELISA. The results show that mice treated with OPG-Fc had significantly higher titer of reactive antibodies than control mice indicating a B cell-mediated immune response.

A)



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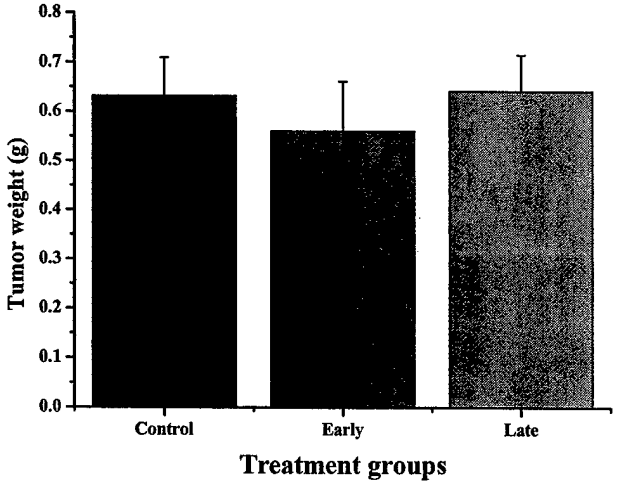


Figure 14. OPG-Fc does not affect 4T1.2 primary tumor growth. Balb/c mice were inoculated with 4T1.2 cells (1 x 10^5) into the mammary fat pad and treated every 3 days with 3 consecutive intraperitoneal injection of PBS (control) or OPG-Fc (5mg/Kg) starting on day 7 (early intervention) or starting on day 14 (late intervention). (A), Tumor volume was measured every 3 days from day 7 to 25 using electronic calipers. (B), At harvest (day 25), primary tumors from each group were weighed. The results show that administration of OPG-Fc under either protocol had not significant effect on the growth (tumor volume and final tumor weight) of 4T1.2 primary tumors.

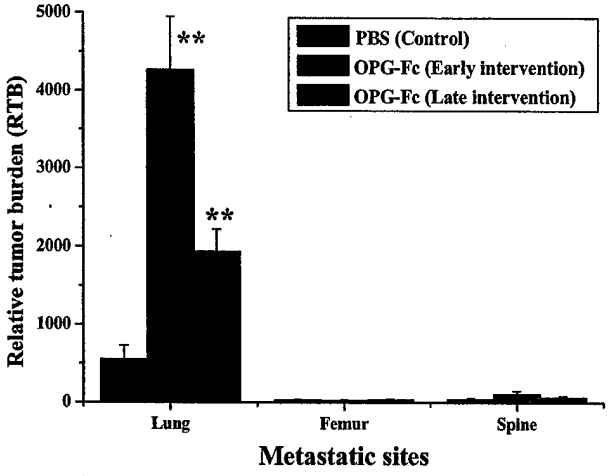


Figure 15. Short-term treatment with OPG-Fc enhances metastatic burden in lung. Balb/c mice were injected with 4T1.2 cells (1 x 10^5) into the mammary fat pad and treated with OPG-Fc of PBS (control) as described in Figure 14 and their lung femurs and spine harvested on day 25 for quantitative measurement of metastatic tumor burden by RTQ-PCR. Animals treated with OPG-Fc (early and late intervention) had significantly higher tumor burden in lung ($p<0.001$) than control mice. Metastatic burden in femurs and spine was low in control mice, most likely due to excessive lung tumor burden requiring early harvest of mice, and was not significantly affected in OPG-Fc-treated mice.

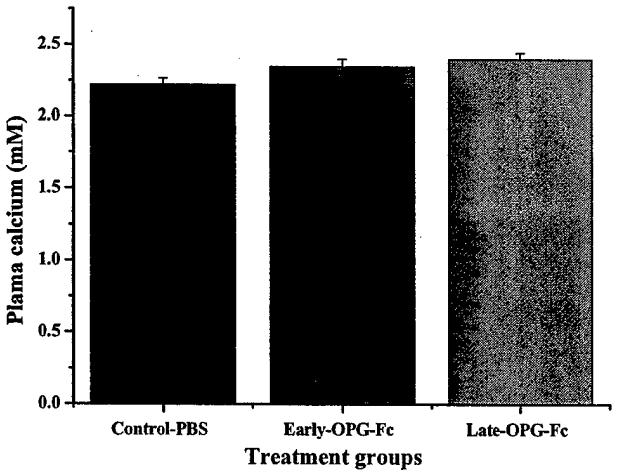


Figure 16. Analysis of plasma calcium level in OPG-Fc-treated mice. Balb/c mice were inoculated with 4T1.2 tumor cells (1 x 10^5) into the mammary fat pad and treated with OPG-Fc as described in Figure 14. Blood samples were collected from control (PBS) and OPG-Fc (early and late intervention) treated mice and their plasma calcium levels measured. Consistent with the low bone tumor burden observed in both groups, calcium levels were normal and not significantly different between groups.

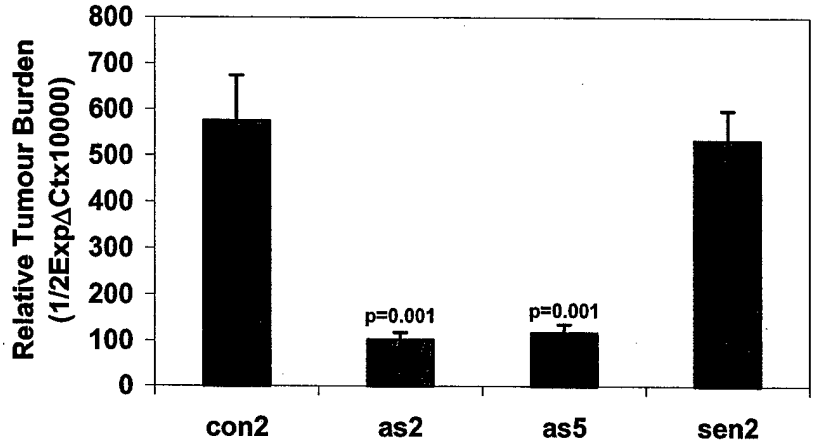


Figure 17. Effect of Antisense PTHrP on Metastatic Tumor Burden. Single cell clones of 4T1.13 tumor cells were transfected with vector (con2), PTHrP sense (sen2) or PTHrP antisense (as2 and as5) and injected into the mouse mammary fat pad (n = 15). Four weeks later, spines were removed and RTQ PCR used to measure metastatic tumor burden. Error bars represent standard error of the mean and p values were calculated using the student t test.

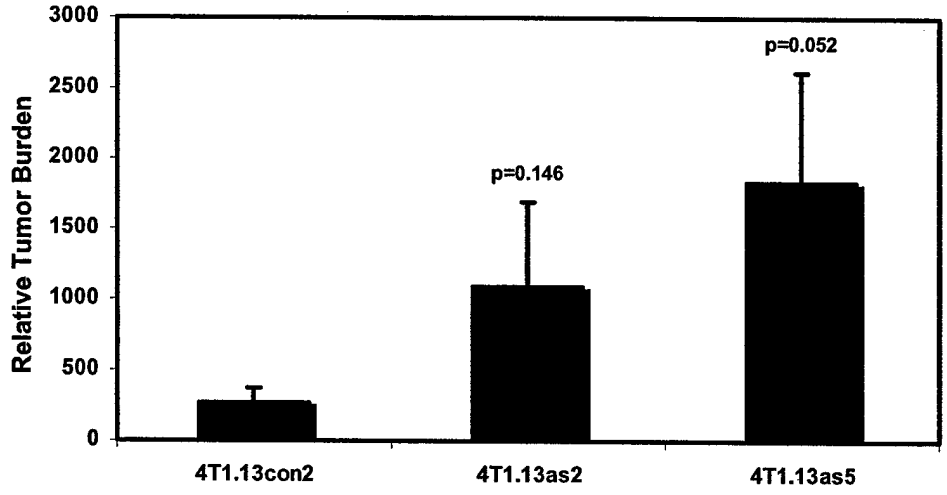


Figure 18. Effect of Antisense PTHrP on Metastatic Tumor Burden. Single cell clones of 4T1.13 tumor cells were transfected with vector (4T1.13con2) or PTHrP antisense (4T1.13as2 and 4T1.13as5) and injected into the mouse mammary fat pad. Four weeks later, spines from mice inoculated with 4T1.13Con2 cells were removed and RTQ PCR used to measure metastatic tumor burden. Spines from mice inoculated with 4T1.13 cells transfected with a PTHrP antisense were harvested and tumor burden measured 7 days later when the primary tumors were the same size as those from control mice. $n = 10$. Error bars represent standard error of the mean and p values were calculated using the student t test.

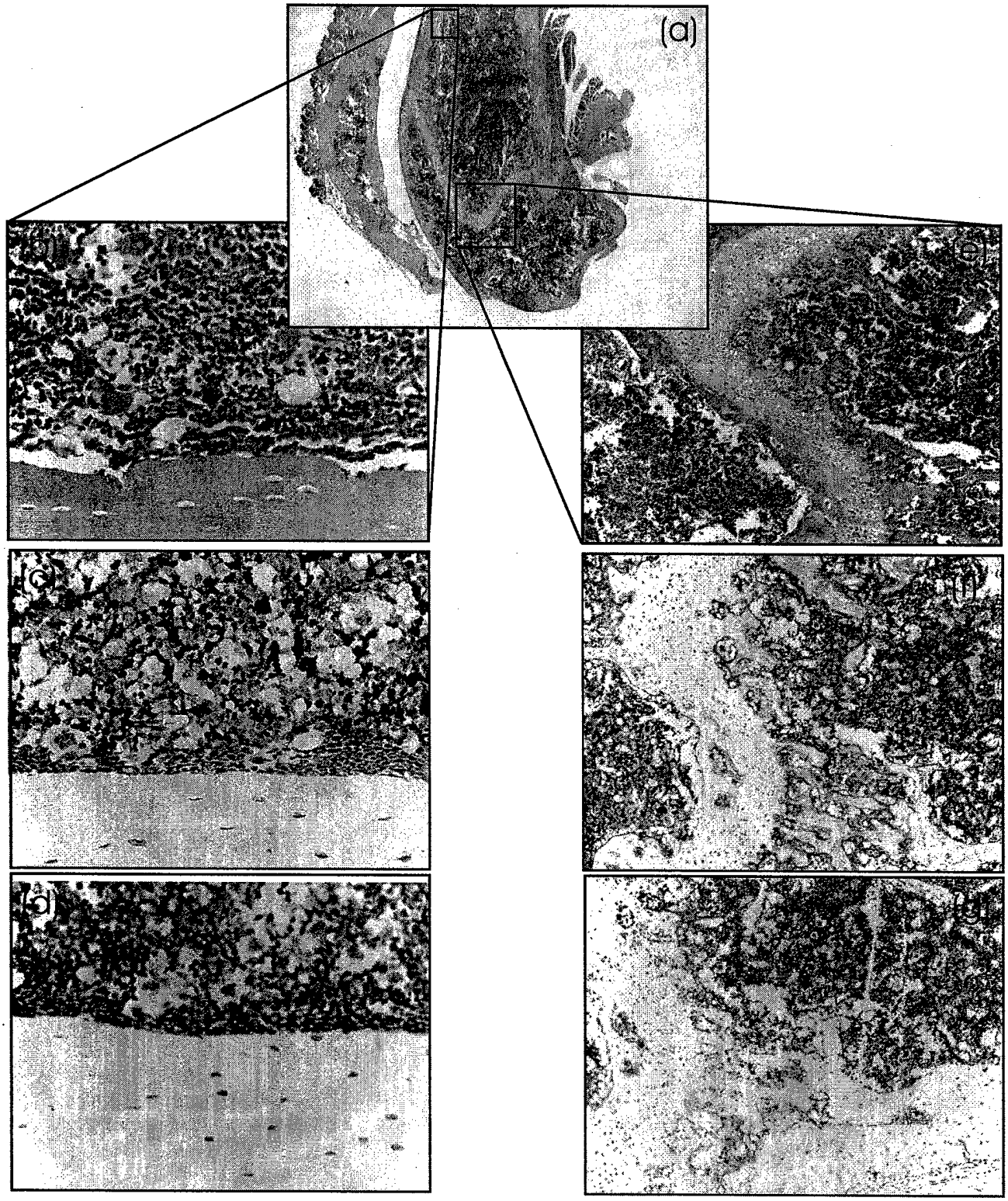


Figure 19. MMP-9 expression in normal femur sections. Femurs from healthy 6-8 week old Balb/c mice were harvested, fixed in formalin and decalcified in EDTA prior to processing for paraffin embedding. Serial sections were stained with hematoxylin and eosin (H&E) or used for immunohistochemical detection of MMP-9 using a polyclonal goat antiserum. (a) H&E stained femur epiphysis. (b) H&E staining of marrow-bone interface. (c) Specific MMP-9 reactivity is seen in haematopoietic cells populating the bone marrow. (D) Absence of specific staining in control sections reacted with pre-immune serum. (e) H&E of the growth plate region. (f) MMP-9 stained serial section localising MMP-9 to the zone of ossification (transition zone from cartilage to bone). (g) negative control reacted with pre-immune serum. Magnification: 2.5X (a), 10X (b-g).

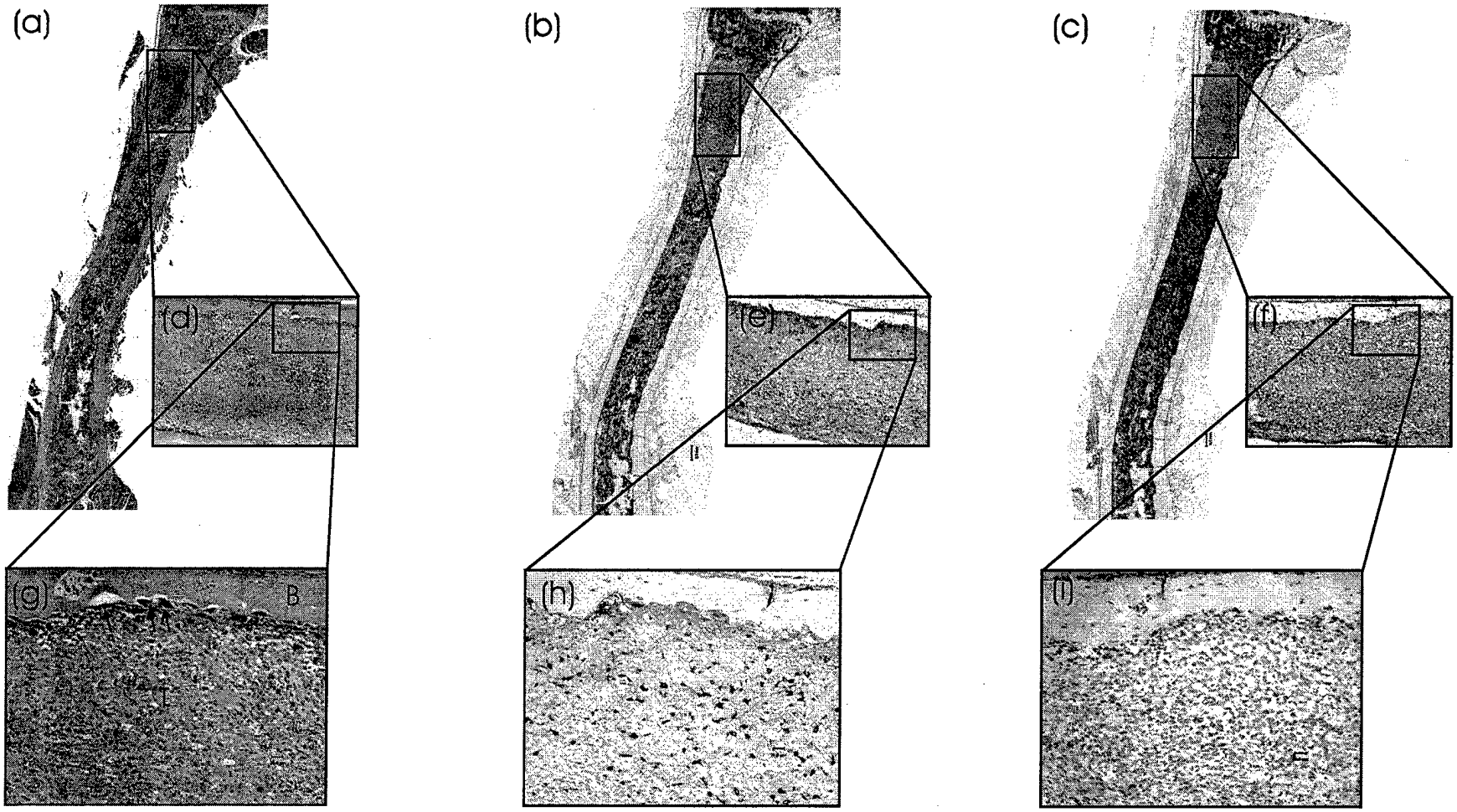


Figure 20. MMP-9 Expression in 4T1.2 femoral metastases. Femurs were harvested 30 days after injection of 4T1.2 tumor cells into the mammary fat pad. Femurs were fixed in formalin, decalcified in EDTA and processed for paraffin embedding. (a) H&E staining shows a large metastatic lesion (t) in the proximal epiphyseal region. (b) serial section reacted with a specific goat polyclonal antiserum directed against MMP-9 following antigen retrieval by trypsin digestion shows MMP-9 immunoreactivity scattered though the marrow and around the metastatic lesion. (C) No specific labeling was detected using a control goat pre-immune serum. (d) H&E stained section. (e) corresponding field stained for MMP-9 demonstrates MMP-9 localization primarily at the periphery of the metastatic lesion. (f) absence of specific staining using pre-immune serum. (g) at higher magnification, H&E of the metastasis-bone interface shows the presence of osteoclasts within resorption lacunae (T = tumor, B = cortical bone, arrows denote osteoclasts and associated resorption lacunae) (h) 20x view of the corresponding stained section, demonstrating MMP-9 inmmunoreactivity within resorption lacunae and in scattered cells within the tumor mass. (i) 20X magnification of the corresponding negative control section. Magnification: 2.5X (a-c), 10X (d-f), 20X (g-i).

Figure 21. MMP-9 expression in 4T1.2 tumor following intratibial injection into wild type Balb/c mice. Tibias were harvested 11 days after direct intratibial injection of 4T1.2 tumor cells, fixed in formalin, decalcified and processed for paraffin embedding. Serial sections were stained for MMP-9 expression following antigen retrieval by trypsin digestion. (a) H&E stained section showing a large 4T1.2 tumor deposit (T) adjacent to bone / cartilage (B/C). (b) MMP-9 stained serial section revealed strong immunoreactivity at the periphery of the tumor adjacent to the bone surface. MMP-9 immunoreactivity is also clearly visible within the cartilage / bone matrix of the growth plate. (c) Absence of specific staining using a control goat pre-immune serum. Magnification: 10X (a-c).

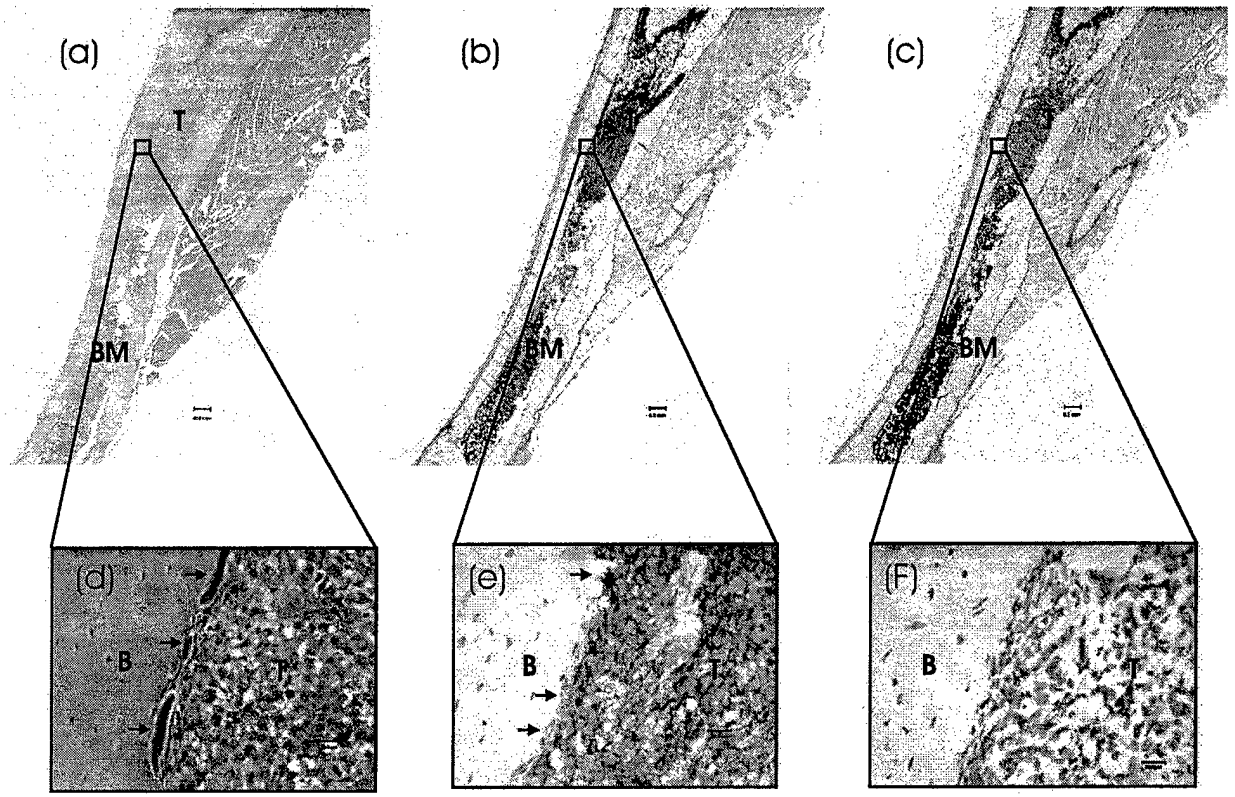


Figure 22. MMP-9 expression in 4T1.2 tumors following intratibial injection into MMP-9 knockout mice. 4T1.2 tumor cells were injected into the proximal marrow space of MMP-9 knockout Balb/c mouse tibias. Tumors were allowed to develop for 11 days prior to harvest. Tibiae were fixed in formalin, and decalcified prior to paraffin embedding. Serial sections were stained for MMP-9 following antigen retrieval by trypsin digestion. (a) H&E stained tibia, demonstrating the tumor mass (T) occupying the upper (proximal) portion of the tibial marrow, adjacent to normal bone marrow (BM). (b) Corresponding serial section stained for MMP-9, showing strong tumor immunoreactivity but absence of specific staining in the surrounding bone. (c) No specific labeling was detected using a control goat pre-immune serum. (d) H&E of tumor-bone interface showing multinucleated osteoclasts within resorption lacunae. (e) Section stained for MMP-9 demonstrating strong immunoreactivity in 4T1.2 tumor cells. (f) Absence of specific staining using control pre-immune serum. *B-cortical bone, T- tumor.* Magnification: 2.5X (a-c), 40X (d-f).

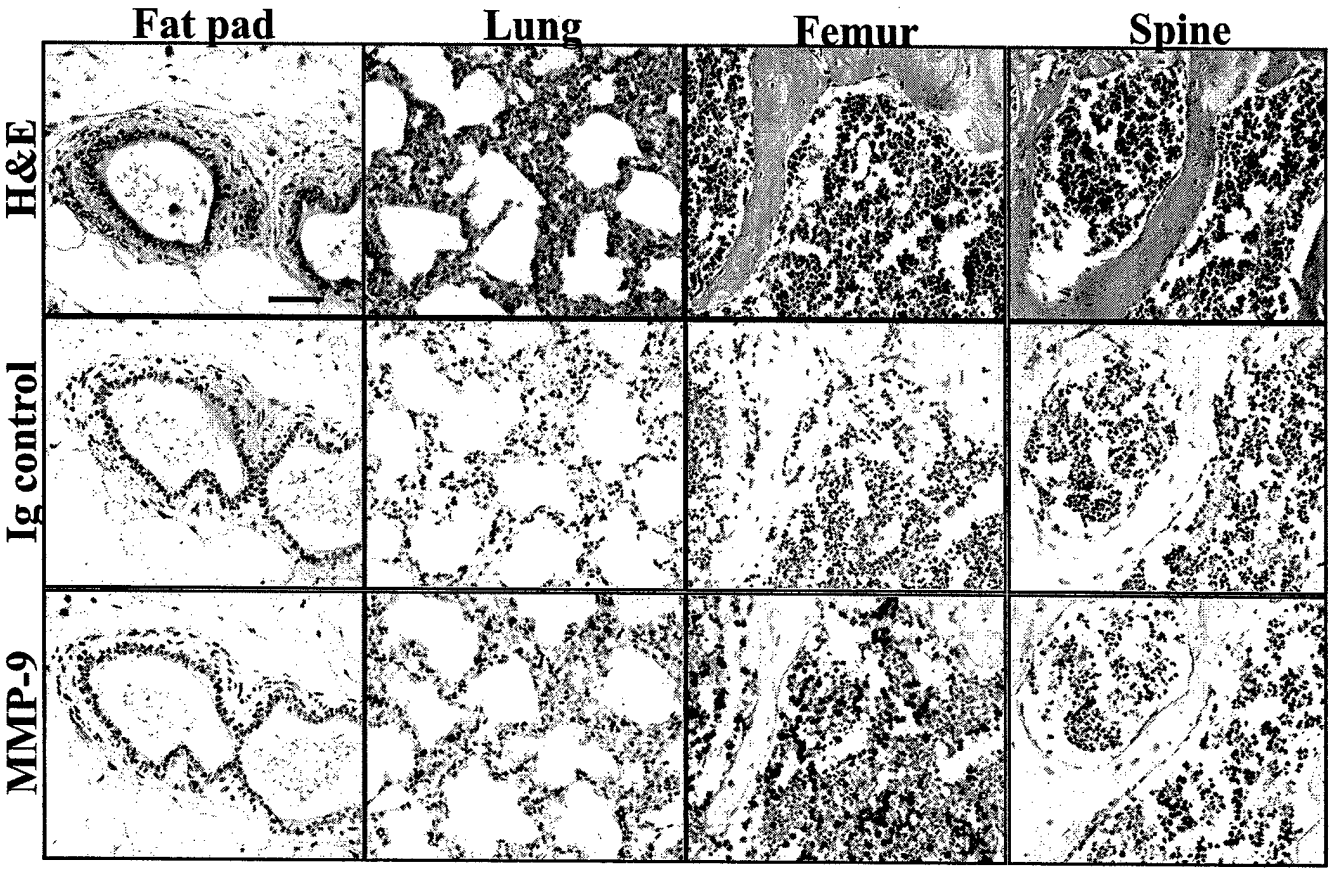
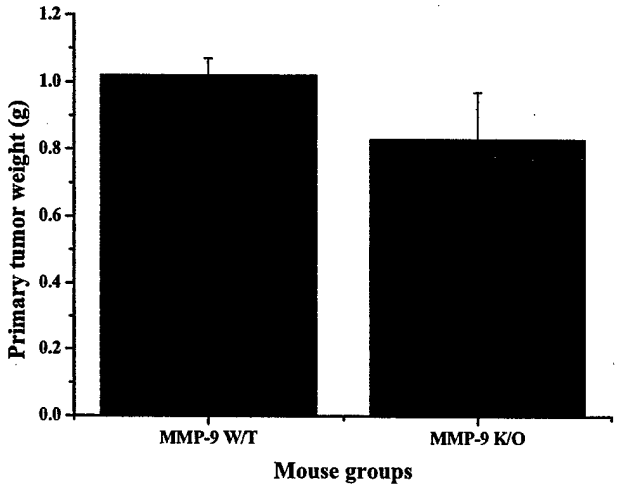


Figure 23. Immunohistochemical detection of MMP-9 in normal wild type mammary fat pad, lung, femur and spine. Paraffin sections of mammary fat pad, lung, femur and spine were obtained from normal wild type Balb/c mice and stained by hematoxylin & eosin (H&E). Corresponding serial sections were stained by standard immunohistochemistry for the detections of MMP-9 using goat anti-mouse polyclonal antibodies (MMP-9) or control preimmune goat antibodies as isotype control (Ig control). Note the scattered MMP-9 positive cells within the lung stroma and the abundance of MMP-9 reactive cells in the bone marrow of femurs and spines. Scale bar = 50um.

a)



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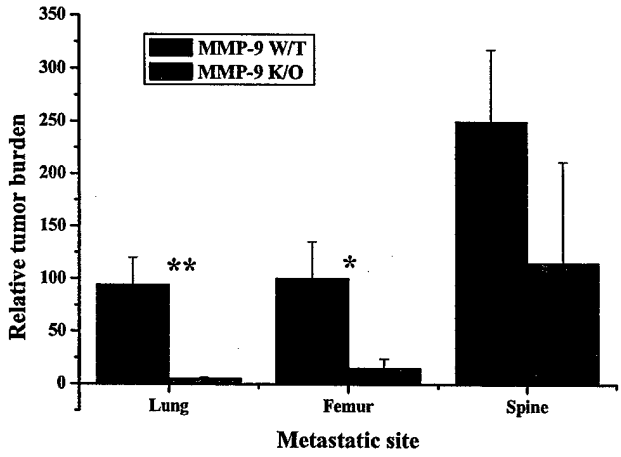

Figure 24. 4T1.2 primary tumor growth and metastatic tumor burden in wild type and MMP-9 knockout mice. 4T1.2 cells (1×10^5) were inoculated into the mammary fat pad of wild type and MMP-9 K/O Balb/c mice and allowed to grow and progress to form spontaneous metastases. Primary tumors were harvested and weighed on day 28 and lung, femurs and spine of metastatic sites processed for quantitative measurement of metastatic burden by RTQ-PCR. (a), Primary tumor weight . 4T1.2 mammary tumor developed to the same size in both wild type and MMP-9 K/.O animals. (b) Metastatic burden in lung, femurs and spine. 4T1.2 metastatic burden was dramatically reduced in lung (*$p<0.01$) and femurs (*$p<0.05$) or MMP-9 K/O mice compared to wild typw control mice. A similar trend was observed in spine but did not reach statistical significance ($p<0.2$).

KEY RESEARCH ACCOMPLISHMENTS

- Development of a real time PCR based assay to quantitate metastatic tumor burden
- Demonstration that PTHrP expression in primary tumors does not correlate with metastasis to bone.
- Demonstration that increased PTHrP expression does not enhance metastasis to bone.
- Demonstration that inhibition of PTHrP expression reduces tumor growth rate in the primary tumor and in metastatic nodules, but does not specifically reduce metastasis to bone.
- Profiling of candidate gene expression in primary tumors by RT-RTQ PCR revealed a number of genes differentially expressed between bone metastatic (4T1.2, lung metastatic (66cl4) and non-metastatic (67NR tumors including IL-1α, IL-6, uPA, MMP-2, MMP-9, RANKL and OPG.
- Profiling of chemokine receptor expression in primary tumors indicates that tumor cells are not directed to bone by chemokines. Expression of the chemokine sdf-1 inhibits primary tumor growth.
- Demonstration that bone homing tumors induce an increase in RANKL and MMP-9 and a decrease in OPG in co-culture with osteoblast cells.
- Demonstration that administration of recombinant OPG reduces metastasis to bone and inhibits tumor cell growth in bone but may enhance soft tissue metastasis.
- Back-crossing of mice lacking functional osteoclasts (beta3 integrin knockout, op/op) or deficient in MMP function (MMP9, MMP12 knockout) onto the Balb/c background.
- Demonstration that the bone metastasizing tumor expresses significantly higher levels of MMP-9 *in vitro* than non-bone metastatic lines.
- Demonstration that co-culture of bone stromal cells and bone metastatic breast tumor cells increases expression of MMP-9 in tumor cells.
- Demonstration that stromal expression of MMP-9 is significantly induced in proximity of tumor cells in lung and bone metastatic lesions. Tumor MMP-9 is up-regulated in bone metastatic lesions as well but not in lung metastases, indicating cross-talk between the tumor and stromal cells.
- Demonstration that stromal MMP-9 contributes to the progression of bone and lung metastases.
- Generated clonal lines of bone metastatic breast tumor cells with reduced MMP-9 expression.

REPORTABLE OUTCOMES

Dr. Michael Tavaria resigned from the fellowship program on March 31, 2003 to take up a position with Applied Biosystems. Dr. Normand Pouliot has taken over as PI for this fellowship.

- Provisional patent for the 4T1.13Con2 bone metastasizing line and the use of RTQ PCR to measure metastasis.

  Tavaria, MD, Tavaria, MD, Moseley, JM, Natoli, A, Lelekakis, M, Hards, D, Stanley, K., Fritzlaff, C., Restall, C, Ho, P., Slavin, J., Martin, TJ, Anderson, RL. PARATHYROID HORMONE RELATED PROTEIN (PTHRP) MODULATES TUMOR GROWTH BUT NOT SPONTANEOUS METASTASIS TO BONE. Submitted to Bone.

- Tavaria, MD, Ross, R, Pouliot, N, Natoli, A, Kendrick, J, Lelekakis, M, Restall, C, Moseley, JM, Dunstan, C and Anderson, RL. OSTEOPROTEGERIN INHIBITS METASTASIS TO BONE IN AN ORTHOTOPIC MOUSE MODEL OF BREAST CANCER METASTASIS. Manuscript in preparation.

- Sloan, E. K., Stanley, K.L., Pouliot, N., Hards, D.K., Moseley, J. M. and Anderson, R.L. αVβ3 INTEGRIN PROMOTES SPONTANEOUS METASTASIS OF BREAST CANCER TO BONE. Submitted to Clinical & Experimental Metastasis.

- Eckhardt, BL, Pouliot, N. and Anderson, RL (2004). INFLUENCE OF THE BONE MICROENVIRONMENT ON BREAST CANCER METASTASIS TO BONE. In Integration/Interaction of Oncologic Growth. Ed. G. Meadows, Kluwer Press, The Netherlands (in press).

- Anderson, RL, Henderson, M, Pouliot, N, Waring, P, Moseley, JM and Sloan EK. STROMAL EXPRESSION OF CAVEOLIN-1 REGULATES BREAST CANCER PROGRESSION. The Fourth International Conference on Cancer-induced Bone Diseases. San Antonio, Texas, December 2003.

- Tavaria, MD, Natoli, A, Kendrick, J, Lelekakis, M, Restall, T, Sloan, E, Hards, D, Ho, P, Martin, TJ, Moseley, JM, Anderson, RL. INHIBITION OF BONE METASTASES IN A MOUSE MODEL OF BREAST CANCER METASTASIS. Abstract and poster presentation at 14th Lorne Cancer Conference, Victoria, Australia. Feb 2002.

- Tavaria, M, Natoli, A, Kendrick, J, Restall, C, Lelekakis, M, Hards, D, Ho, P, Martin, TJ, Moseley, J, Anderson, RL. INTERACTIONS BETWEEN METASTATIC BREAST TUMOR CELLS, STROMAL CELLS AND OSTEOCLASTS IN THE BONE MICROENVIRONMENT. Platform presentation at the 1Xth International Congress of the Metastasis Research Society, Chicago, September, 2002.

- Tavaria, MD, Natoli, A, Restall, C, Kendrick, J, Lelekakis, M, Hards, D, Ho, P, Martin, TJ, Moseley, J, Anderson, RL. BREAST TUMOR STROMAL CELL INTERACTIONS IN THE BONE. Abstract and poster presentation at the Department of Defense Breast Cancer Research Program Era of Hope Meeting, Orlando, September 2002.

- Tavaria, MD, Natoli, A, Lelekakis, M, Ho, P, Hards, D, Martin, TJ, Moseley, JM, Anderson, RL. INHIBITION OF BREAST CANCER METASTASIS TO BONE IN AN ORTHOTOPIC MOUSE MODEL. Abstract and platform presentation at 4th International Conference of Cancer Induced Bone Diseases, Awaji Shima, Japan. Nov 2001.

CONCLUSIONS

The development of the orthotopic spontaneous breast cancer metastasis to bone model has allowed us to address issues in bone metastasis not adequately addressed with previous animal models. In agreement with recent clinical data, we have shown that PTHrP does not enhance metastasis to bone, but it does exert an effect on growth of breast cancer cells. This may impact on clinical management of metastatic breast cancer where use of neutralizing antibodies against PTHrP is being considered as a treatment modality.

On the other hand, we have demonstrated the value of osteoprotegerin in reducing metastatic burden in bone. These experiments were recently expanded to use the new more potent form of OPG provided by Amgen (OPG-Fc). Whilst the interpretation of the results has been complicated by the development of a B cell-mediated immune response to the human fusion protein, ongoing experiments in immunocompromised mice will provide indications of the therapeutic potential of this compound for the treatment of breast malignancy in human. However, our dat indicating an increase in lung metastasis in OPG-Fc-treated mice raise a major concern that needs to be confirmed. OPG is undergoing Phase 1 clinical trials and appears to be a promising modality. In contrast, the other agents commonly used to block osteoclast action, the bisphosphonates, do not appear to be effective in reducing tumor burden in bone in our model. However, it remains to be seen if they reduce osteolysis induced by tumor cells.

Our co-cultures of bone metastasizing cells and stromal cells have revealed the ability of the tumor cells to interrupt the signaling between osteoblasts and osteoclasts through alterations in levels of expression of RANKL and OPG. Although we have incurred some delays with our *in vivo* immunohistochemical analysis of RANKL due to ineffective antibody provided by R&D Systems (see appendix), we are confident that ongoing experiments testing the efficacy of murine RANK-Fc (also provided by Amgen) in breast cancer metastasis to bone and completion of the RANKL IHC analysis will support our *in vitro* findings and lead to a high impact publication.

Our finding that expression of β3 integrin in a line that only metastasise to lung can cause the line to metastasise to bone as well, reveals the importance of αvβ3 in bone metastasis. Recent testing of a novel integrin β3 inhibitor in collaboration with a pharmaceutical industry partner indicates that the drug is well tolerated but its impact on breast cancer metastasis to bone awaits analysis of tumor burden in bone of treated animals.

Our co-cultures and in vivo assays have also revealed regulation of MMP9 in the tumor cells. In analyzing our current data, it appears that the tumor cells utilize stromal MMP9 during growth in bone and that tumor-derived MMP-9 is also significantly upregulated by bone stromal cells *in vitro* and *in vivo*. Our in vivo results clearly demonstrate that stroma-derived MMP-9 plays a critical role in the development of both bone and lung metastasis and justifies the use of specific MMP-9 inhibitors for further clinical studies. Our preliminary results from downregulation of MMP-9 in tumor cells suggest that tumor-derived MMP-9 may be particularly important for the development of bone metastases.

We take the opportunity to thank the Department of Defense Breast Cancer Research Program for their valuable financial support over the past 3 years and look forward to publicizing our findings through conferences, publications and several collaborations established in the course of this Award.

BIBLIOGRAPHY:

1. Kakonen, S. M. and Mundy, G. R. Mechanisms of osteolytic bone metastases in breast carcinoma. Cancer, *97:* 834-839, 2003.
2. Brooks, B., Bundred, N. J., Howell, A., Lang, S. H., and Testa, N. G. Investigation of mammary epithelial cell-bone marrow stroma interactions using primary human cell culture as a model of metastasis. Int J Cancer, *73:* 690-696, 1997.
3. Hahn, T., Or, R., Segall, H., and Karov, Y. Human bone marrow-derived mitogenic stimulation selective for breast carcinoma and neuroblastoma cells. Int J Cancer, *78:* 624-628, 1998.
4. Rosol, T. J., Tannehill-Gregg, S. H., LeRoy, B. E., Mandl, S., and Contag, C. H. Animal models of bone metastasis. Cancer, *97:* 748-757, 2003.
5. Yoneda, T. Cellular and molecular basis of preferential metastasis of breast cancer to bone. J Orthop Sci, *5:* 75-81, 2000.
6. Harms, J. E. and Welch, D. R. MDA-MB-435 human breast carcinoma metastasis to bone. Clin Exp Metastasis, *20:* 327-334, 2003.
7. Lelekakis, M., Moseley, J. M., Martin, T. J., Hards, D., Williams, E., Ho, P., Lowen, D., Javni, J., Miller, F. R., Slavin, J., and Anderson, R. L. A novel orthotopic model of breast cancer metastasis to bone. Clin Exp Metastasis, *17:* 163-170, 1999.
8. Thomas, R. J., Guise, T. A., Yin, J. J., Elliott, J., Horwood, N. J., Martin, T. J., and Gillespie, M. T. Breast cancer cells interact with osteoblasts to support osteoclast formation. Endocrinology, *140:* 4451-4458, 1999.
9. Guise, T. A., Yin, J. J., Thomas, R. J., Dallas, M., Cui, Y., and Gillespie, M. T. Parathyroid hormone-related protein (PTHrP)-(1-139) isoform is efficiently secreted in vitro and enhances breast cancer metastasis to bone in vivo. Bone, *30:* 670-676, 2002.
10. Wysolmerski, J. J., Dann, P. R., Zelazny, E., Dunbar, M. E., Insogna, K. L., Guise, T. A., and Perkins, A. S. Overexpression of parathyroid hormone-related protein causes hypercalcemia but not bone metastases in a murine model of mammary tumorigenesis. J Bone Miner Res, *17:* 1164-1170, 2002.
11. Henderson, M., Danks, J., Moseley, J., Slavin, J., Harris, T., McKinlay, M., Hopper, J., and Martin, T. Parathyroid hormone-related protein production by breast cancers, improved survival, and reduced bone metastases. J Natl Cancer Inst, *93:* 234-237, 2001.
12. Bendre, M. S., Gaddy-Kurten, D., Mon-Foote, T., Akel, N. S., Skinner, R. A., Nicholas, R. W., and Suva, L. J. Expression of interleukin 8 and not parathyroid hormone-related protein by human breast cancer cells correlates with bone metastasis in vivo. Cancer Res, *62:* 5571-5579, 2002.
13. Muller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S. N., Barrera, J. L., Mohar, A., Verastegui, E., and Zlotnik, A. Involvement of chemokine receptors in breast cancer metastasis. Nature, *410:* 50-56, 2001.
14. Taube, T., Elomaa, I., Blomqvist, C., Beneton, M. N., and Kanis, J. A. Histomorphometric evidence for osteoclast-mediated bone resorption in metastatic breast cancer. Bone, *15:* 161-166, 1994.
15. Yasuda, H., Shima, N., Nakagawa, N., Yamaguchi, K., Kinosaki, M., Goto, M., Mochizuki, S. I., Tsuda, E., Morinaga, T., Udagawa, N., Takahashi, N., Suda, T., and Higashio, K. A novel molecular mechanism modulating osteoclast differentiation and function. Bone, *25:* 109-113, 1999.

16. McHugh, K. P., Hodivala-Dilke, K., Zheng, M. H., Namba, N., Lam, J., Novack, D., Feng, X., Ross, F. P., Hynes, R. O., and Teitelbaum, S. L. Mice lacking beta3 integrins are osteosclerotic because of dysfunctional osteoclasts. J Clin Invest, *105:* 433-440, 2000.
17. Duffy, M. J., Maguire, T. M., Hill, A., McDermott, E., and O'Higgins, N. Metalloproteinases: role in breast carcinogenesis, invasion and metastasis. Breast Cancer Res, *2:* 252-257, 2000.
18. La Rocca, G., Pucci-Minafra, I., Marrazzo, A., Taormina, P., and Minafra, S. Zymographic detection and clinical correlations of MMP-2 and MMP-9 in breast cancer sera. Br J Cancer, *90:* 1414-1421, 2004.
19. Ranuncolo, S. M., Armanasco, E., Cresta, C., Bal De Kier Joffe, E., and Puricelli, L. Plasma MMP-9 (92 kDa-MMP) activity is useful in the follow-up and in the assessment of prognosis in breast cancer patients. Int J Cancer, *106:* 745-751, 2003.
20. Ohshiba, T., Miyaura, C., Inada, M., and Ito, A. Role of RANKL-induced osteoclast formation and MMP-dependent matrix degradation in bone destruction by breast cancer metastasis. Br J Cancer, *88:* 1318-1326, 2003.
21. Tester, A. M., Ruangpani, N., Anderso, R. L., and Thompson, E. W. MMP-9 secretion and MMP-2 activation distinguish invasive and metastatic sublines of a mouse mammary carcinoma system showing epithelial-mesenchymal transition traits. Clin Exp Metastasis, *18:* 553-560, 2000.

other R+D Systems products- anti-mouse OPG matched antibody pair for ELISA(Cat#MAB4591/BAF459) and anti-mouse OPG Cat#BAF459 for IHC. However, I cannot afford to spend time and money on antibodies that turn out faulty. Before I purchase these products I would appreciate a test sample so that I can confirm the product works. Then I would be happy to purchase these antibodies immediately and continue to use them in the future. Currently I just cannot afford the risk of such valuable product being faulty and I need to test a sample first.
Many thanks for your help thus far and I look forward to your prompt reply,
Cheers,
Richard Ross,
Cancer Biology, Peter
MacCallum Cancer Centre.

-----Original Message-----
From: Daryn Metti [mailto:daryn@biosci.com.au]
Sent: Wed 21/07/2004 8:47 AM
To: Ross Richard
Cc:
Subject: RE: AF462; R+D Systems; Complaint # 0312-36; DM

Dear Ross

R&D have finally worked out the problem, as mentioned below;
I am very sorry this took so long. In reconstituting a vial of the Lot CKN021091, we found that the vial did not go into solution well. Perhaps the customer also did not have the antibody go into solution fully and this may have been contributing to the lack of success with the antibody. We have bottled Lot CKN034061 and it has passed QC. We reconstituted and tested a vial of this bottling in IHC on mouse thymus and working dilution we recommend is from 1.7 to 15 ug/ml.
Do you think your customer is interested in trying anther vial?
Please let me know and I will send a N/C authorization for a vial of the Lot CKN034061. Sorry we did not discover this problem earlier and thank you for your patience.
Please advise if you want the replacement vial.

Thanks & Regards
Daryn Metti
General Manager
Bioscientific Pty Ltd

-----Original Message-----
From: Ross Richard [mailto:richard.ross@petermac.org]
Sent: Monday, 24 May 2004 4:50 PM
To: Daryn Metti
Subject: AF462; R+D Systems; Complaint # 0312-36; DM

Hi Daryn,
Yes, I did buy the Goat anti-RANKL antibody for use in mouse. I tried the antibody on a human sample to assess whether it mayhave been a human specific and not mouse specific antibody, only after I exhuasted all other avenues of investigation. So , these results together with the many others I have generated on mouse tissue, suggest that the goat anti-mouse RANKL does not see mouse or human RANKL

via IHC, no matter what the antigen retrieval method or concentration.

Thanks,

Rich Ross,
PMCI
-----Original Message-----
From: Daryn Metti [mailto:daryn@biosci.com.au]
Sent: Mon 24/05/2004 9:53 AM
To: Ross Richard
:
Subject: RE: AF462; R+D Systems; Complaint # 0312-36; DM

Dear Ross
R&D has said/asked;
We are sorry to hear that the antibody did not work successfully for your customer. Your customer states that they have used both mouse and human samples. There is not a high degree of homology between mouse and human TRANCE (amino acid identity of 64%). They do want an antibody to mouse, correct?

Thanks & Regards

Daryn Metti
Sales & Marketing Manager
Bioscientific Pty Ltd

---Original Message-----
From: Ross Richard [mailto:richard.ross@petermac.org]
Sent: Thursday, 20 May 2004 6:42 PM
To: Daryn Metti
Subject: RE: AF462; R+D Systems; Complaint # 0312-36; DM

Hi Daryn,
I write in reply to some phone calls received about the replacement R+D Systems Goat anti-mouse RANKL (AF-462) pAb I received. I was given the original antibody (AF-462), which we confirmed did not work to specifications. It was agreed by R+D that they would give me a replacement of this antibody, which I have received and tested. Unfortunately, again I cannot generate a positive result from this antibody. I have tried it on mouse spine/femur (cryoembedded and paraffin embedded), mouse lymph nodes paraffin), normal human breast (paraffin) and cancerous human breast (paraffin) to no avail. In the paraffin embedded sections I have tried microwave/citrate buffer pH10 and pH6, trypsin and pepsin, and in cryoembedded sections I did not use any Ag retrieval. Still none of these techniques worked. I am now running out of time and can strongly suggest that this antibody does not see mouse RANKL, considering I followed the exact suggested protocol (pH6 citrate buffer/ microwave). I asked a post-doc here at Peter Mac to try using it on the same sections, again he had no specific staining and he is highly experienced in IHC methods. As this antibody has not given any positive result with any section (even a positive control given in the R+D website), I cannot persist in testing it and wasting time/money, neither of which I have vast amounts. Is a refund/ replacment with a different antibody possible, considering the current performance, or lack thereof of this product.
Many thanks,

Richard Ross,
Cancer Biology, Peter MacCallum Cancer Centre,
Melbourne.

Appendix

From: Ross Richard
Sent: Monday, 26 July 2004 10:44 AM
To: Pouliot Normand
Subject: FW: AF462; R+D Systems; Complaint # 0312-36; DM

-----Original Message-----
From: Daryn Metti [mailto:daryn@biosci.com.au]
Sent: Mon 26/07/2004 9:36 AM
To: Ross Richard
Cc:
Subject: RE: AF462; R+D Systems; Complaint # 0312-36; DM

Dear Ross

R&D has said;
I am sorry that you have experienced these difficulties. I am glad you are willing to try the replacement vial of the anti-mouse TRANCE (RANK L).

Regarding samples of the OPG antibodies: Do you think it would be acceptable if I provided you with the QC results for these antibodies before you purchased them so that you could analyze our results. Hopefully this would provide the evidence that these products will work as stated. I need to double-check on the BAF459, according to the insert the antibody has not been validated for IHC. Also, please reassure your customer that we will fully support these products. I can understand their frustration, since they spent so much time trouble shooting the anti-RANK L Ab on their own.

Please let me know what your customer would like to do at this point.

Regards
Daryn

-----Original Message-----
From: Ross Richard [mailto:richard.ross@petermac.org]
Sent: Thursday, 22 July 2004 5:07 PM
To: Daryn Metti
Subject: RE: AF462; R+D Systems; Complaint # 0312-36; DM

Dear Daryn,

Thankyou for your work in liasing with R+D Systems on my behalf. I have re-evaluated my experimental plans due to the performance of this anti-mouse RANKL pAb.R+D Systems' offer of a replacement vial of anti-mouse RANKL Lot #CKN034061 is kind and I wish to accept this replacement as soon as possible. At this point, I wish to express that the amount of time, reagents and samples I used in testing the faulty batch was considerable. Myself and a post-doc expert in IHC spend many weeks assessing 7 different antigen retrieval methods, multiple fixation and sectioning methods only to find that none of them worked at all. I am counting on these results for a submission due very soon, and the failure of the previous RANKL antibody has made things very difficult and set us back approximately 3 months.

I also wish to purchase